Host resistance mechanism and fungal infection strategies in the *Botrytis cinerea*-tomato interaction

Yaohua You

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Propositions

1. The evidence provided by Itkin et al. (2013) for proposing that GAME2 catalyzes the final glycosylation step in α-tomatine biosynthesis is insufficient and misleading. (this thesis)

2. RNA-Seq studies that compare the transcriptome of different plant species are poorly informative and should be discouraged. (this thesis)

3. Future developments in molecular biology can eventually free human beings as well as plants from most of the known diseases.

4. Analagous to DNA and RNA sequence providers, private companies offering genetic modification services could facilitate academic research on plants and fungi.

5. Taking good decisions is more important than only making efforts, but whether a decision is good only becomes apparent after efforts are made.

6. Testing the hypothesis of a supervisor is more likely to generate positive results, but contributes less to scientific independence than testing one's own hypothesis.

Propositions belonging to the thesis, entitled

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Yaohua You

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Thesis

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Chapter 1

General introduction

The causal agent of grey mould disease, *Botrytis cinerea*

The fungal plant pathogen *Botrytis cinerea*, known as grey mould, is one of the most comprehensively studied necrotrophs. It can infect more than 1400 plant species including economically important fruit and vegetable crops as well as ornamentals (AbuQamar et al., 2017; Dean et al., 2012; Elad et al., 2016; Veloso & van Kan, 2018). *B. cinerea* can infect and grow on damaged or senescing tissues by secreting enzymes and toxic compounds to degrade and kill host cells (van Kan, 2006). This generalist pathogen can cause a broad range of symptoms across different plant species in humid environments. The most typical symptoms include soft rotting by decomposing plant biomass as well as a rapid appearance of grey conidiophores on leaves and soft fruits (AbuQamar et al., 2017; Williamson et al., 2007). Subsequently, macroconidia produced on the conidiophores can serve in dispersal and become sources of secondary infection. Under harsh conditions, mycelia of *B. cinerea* can form sclerotia for survival. Sclerotia play a role in sexual reproduction and in the persistence of this plant pathogen in soil (Amselem et al., 2011). Altogether, grey mould disease caused by *B. cinerea* leads to significant economic losses worldwide, exceeding two thousand million €/year (Dean et al., 2012). Chemical control based on synthetic fungicides remains the most common strategy to control *B. cinerea* epidemics (Fillinger and Walker, 2016). However, application of fungicides is generally expensive and ineffective, because fungicide resistance in *B. cinerea* multidrug resistant (MDR) strains have been reported (Kretschmer et al., 2009 ; Leroch et al., 2011; Leroch et al., 2013), and also raises environmental concerns (Petrasch et al., 2019; Feng et al., 2020). Therefore, equipment of host plants with defense mechanisms or the elimination of genes promoting fungal infection (susceptibility genes) may enable sustainable resistance breeding (Pavan et al., 2010; Sun et al., 2017), which requires a better understanding of the plant-pathogen interaction.

Wild tomato species as genetic resources to explore the resistance mechanisms against *B. cinerea*

Among the economically important hosts of *B. cinerea*, tomato *Solanum lycopersicum* is particularly susceptible to this pathogen. *B. cinerea* can cause heavy losses to tomato production (ten Have et al., 2007; Ji et al., 2012; Smith et al., 2014). Fortunately, wild *Solanum* species represent a rich source of germplasms with different levels of resistance to abiotic and biotic stresses (Egashira et al., 2000; Hanson et al., 2000; Liu et al., Mutschler et al., 1996; 2015; Razali et al., 2018; Rosello et al., 1999; Rose et al., 2007; Shinde et al., 2018; ten Have et al., 2007). Accessions of some wild *Solanum* species that are closely related to *S. lycopersicum* are crossable with cultivated tomato and consequently, introgressions from wild accessions have been performed aiming to improve the resistance to plant diseases (Barbieri et al., 2010; Finkers et al., 2007; Hanson et al., 2000), tolerance to abiotic stress (Foolad et al., 2003; Kissoudis et al., 2015) and agronomic traits (Lecomte et al., 2004; Mangat et al., 2021; Schmalenbach et al., 2009). One of the wild *Solanum* accessions, *S. habrochaites* LYC4 exhibited a high level of partial resistance to *B. cinerea* (ten Have et al., 2007; Finkers et al., 2007). Disease parameters, such as infection frequency and lesion growth rate in leaves and stem segments were significantly reduced in LYC4, as compared with cultivated tomato cv. Moneymaker (MM). A population of 30 introgression lines (ILs) was developed that covers around 95% of the LYC4 genome in the genetic background of MM, and ten quantitative trait loci (QTLs) derived from LYC4 were identified that confer stem resistance to *B. cinerea* (Finkers et al., 2007). However, all these QTLs individually displayed minor quantitative effects in resistance against *B. cinerea* indicating that multiple QTLs need to be combined to achieve a high level of resistance (Finkers et al., 2007). Genetic loci conferring resistance to *B. cinerea* were also mapped in other wild accessions including *S. lycopersicoides* (Davis et al., 2009) and *S. neorickii* (Finkers et al., 2008). These studies demonstrated the importance of wild accessions and the derived IL populations as tools to improve and explore the resistance to *B. cinerea*. However, there are drawbacks of using such IL populations. The introgressions are large segments of chromosomes from LYC4 which in some cases not only cause failure to set seeds (on the long arm of Chromosome 5) but also contain a large number of genes. Due to inversions or species-specific gene regions, the *S. lycopersicum* and *S. habrochaites* chromosomes do not pair along the entire genome (Wolters et al., 2015) and make it problematic to further reduce the size of the introgressions through crossing to pinpoint individual genes responsible for the quantitative resistance. Moreover, LYC4 plants and some of its ILs displayed aberrant morphology as compared with MM, such as a woody stem, waxy leaf surface and intumescence (a physiological disorder). These traits not only restrict the quality of

the plant material for disease assays, but also make it difficult to decipher whether the resistance phenotypes are caused by active defense responses or tissue architecture.

Therefore, optimization of the growth condition of LYC4 and ILs is important to provide good quality material which is essential to explore resistance traits. Treatment with UV-B light in a greenhouse can significantly reduce the severity of leaf intumescence (**Figure 1**).

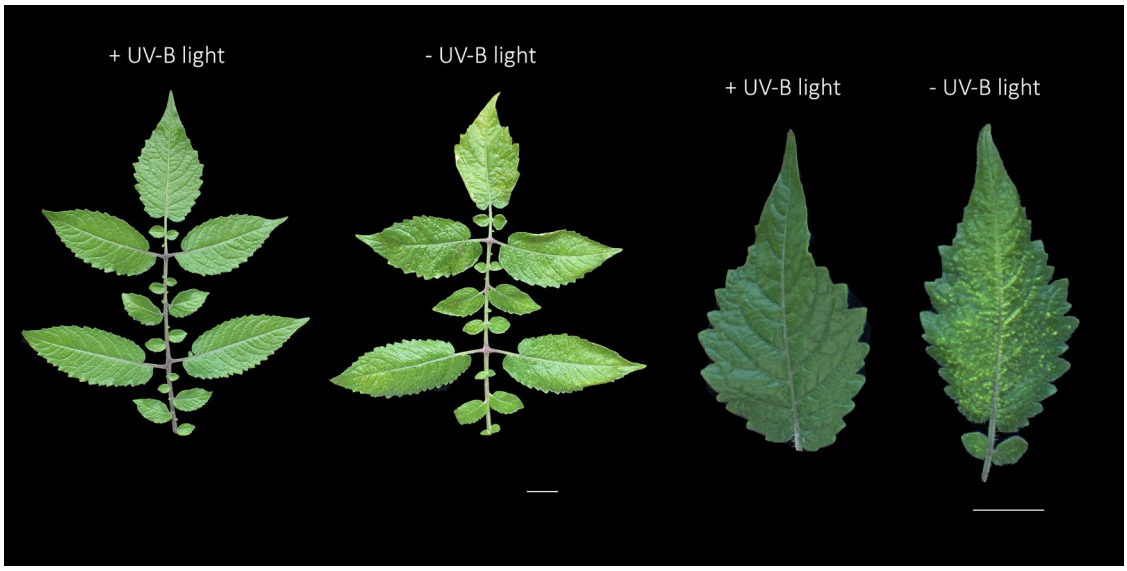

Figure 1. Effect of UV light treatment on LYC4 in preventing the occurrence of leaf intumescence. Scale bar indicates 2 cm.

Role of programmed cell death in the interaction between *B. cinerea* and host plants

Studies regarding plant-necrotroph interactions highlighted the crucial role of programmed cell death (PCD) in plant susceptibility (Faris and Friesen, 2020; Wen, 2013). In general, *B. cinerea* infection can trigger an oxidative burst and subsequently induce plant necrosis. The elicitation of PCD known as hypersensitive response (HR) upon inoculation with a biotrophic pathogen normally leads to host resistance because of the deprivation of water and nutrients that biotrophs rely on to sustain colonization of host plants (Glazebrook, 2005). Unlike biotrophic pathogens, *B. cinerea* as a typical necrotrophic pathogen actively induces and takes advantage of host PCD to facilitate infection (Govrin and Levine, 2000; Mayer et al., 2001).

Besides HR, a different type of PCD that is relevant to *B. cinerea*-host interactions is the apoptotic cell death. A tomato type 2 metacaspase gene sharing protein domain features with mammalian caspase which is known to mediate apoptosis, was induced during PCD in *B. cinerea*-infected leaves (Hoeberichts

et al., 2003). This observation indicated the important role of induction of PCD in successful *B. cinerea* infection and was further supported by the study of metacaspase mutants in *Arabidopsis thaliana*. Mutations in *A. thaliana* type 2 metacaspases which positively regulate PCD led to increased resistance against *B*. *cinerea,* whereas mutants of type 1 metacaspases which negatively regulate PCD, were more susceptible than the wild type (van Baarlen et al., 2007). Furthermore, transgenic plants expressing human, nematode, or baculovirus genes which negatively regulate apoptosis showed enhanced resistance to necrotrophic pathogens *Sclerotinia sclerotiorum* and *B. cinerea* (Dickman et al., 2001). Although histological evidence for the induction of apoptosis during *B. cinerea* infection is lacking, it has been reported that PCD induced by *B. elliptica* in lily showed hallmark features of apoptosis, such as cytoplasmic shrinkage and DNA laddering (van Baarlen et al., 2004).

Autophagy ("self-eating" in Greek) is another pathway for PCD in eukaryotic cells, which is involved in degradation and recycling of unnecessary or dysfunctional cellular components as well as mediating autophagic cell death (Bozhkov, 2018; Veloso and van Kan, 2018). In general, autophagy in plants has been reported to be required for nutrient remobilization during starvation and senescence and also participates in immunity, particularly against necrotrophic pathogen (Bassham, 2007; Zhou et al., 2014). The necrotrophic fungus *S. sclerotiorum*, which is closely related to *B. cinerea*, can induce apoptotic cell death by secreting oxalic acid (OA) (Kim et al., 2008). By contrast, an OA-defective *S. sclerotiorum* mutant generated using UV radiation (Godoy et al., 1990), elicited early, spatially restricted autophagic cell death during tomato infection (Kabbage et al., 2013). Moreover, the inhibition of autophagy either by using Arabidopsis mutants lacking the autophagy-related (ATG) genes *ATG7* and *ATG8*, or by exogenous application of chemical autophagy inhibitors impeded plant resistance and restored the virulence of OA mutants (Kabbage et al., 2013). Recent studies with genetically defined OA mutants in *S. sclerotiorum* revealed that the acidification rather than cell death suppression is required for disease progress (Xu et al., 2018). During *B. cinerea*-Arabidopsis interaction, induction of autophagy-related genes and formation of autophagosomes were reported and Arabidopsis knockout mutants in the genes *ATG5*, *ATG7*, and *ATG18* displayed increased susceptibility towards *B. cinerea* (Lai et al., 2011). However, the involvement of autophagy in the host-*B. cinerea* interaction has not been validated in plants other than Arabidopsis or with different Arabidopsis *atg* mutants by other groups. Besides, there is evidence that plant ATG proteins are not only required for autophagic cell death but might also be involved in other physiological processes. Arabidopsis ATG18a has been reported to physically interact with WRKY33 which is an important transcription factor regulating hormonal and metabolic responses (Birkenbihl et al., 2012; Lai et al., 2011). Recently, ATG8 was also shown to interact with transporter protein ABS3 to modulate plant senescence

independent of its autophagy function (Jia et al., 2019). In summary, the exact role of autophagy in cell death processes during *B. cinerea* infection, either pro-survival or pro-death, remains unclear. In light of the importance of host PCD in resistance to *B. cinerea*, investigations are needed to further shed light on the connection of autophagy to host PCD in plant resistance against *B. cinerea*.

Based on the studies of plant PCD and *B. cinerea* pathogenicity, Veloso and van Kan (2018) proposed that in order to achieve successful colonization of host plants, *B. cinerea* needs to suppress autophagic cell death at an early infection stage and otherwise the induction of autophagy will result in plant resistance. Subsequently, the fungus triggers apoptosis to facilitate lesion formation and expansion in a later stage.

Tolerance to plant defense compounds plays a role in full virulence of *B. cinerea*

Secondary metabolites with antimicrobial activities have long been considered to play an important role in plant defense against pathogens (Piasecka et al., 2015). Such metabolites can have chemically very diverse structures, but they were conveniently classified as phytoanticipins and phytoalexins, based on when they are synthesized (Paxton, 1981; VanEtten et al., 1994). Phytoanticipins were defined as constitutive defense compounds present at high concentration in plant cells before the invasion of plant pathogens, for instance, saponins. On the other hand, phytoalexins are antimicrobial compounds either *de novo* synthesized or activated in response to pathogen challenge, such as camalexin.

Saponins are glycosylated triterpenoid, steroid or steroidal alkaloid molecules with a wide range of antimicrobial properties implicated as phytoanticipins because of their constitutive accumulation in plant tissues (Keukens et al., 1995; Osbourn, 1996). In tomato, the major saponin is α-tomatine with well-documented *in vitro* antimicrobial and anti-insect activity (Sandrock and VanEtten, 1998). The structure of α-tomatine consists of the aglycon tomatidine attached to a tetrasaccharide referred to as lycotetraose. The antifungal activity of α-tomatine is primarily achieved through the disruption of the fungal membrane upon the complexing with membrane sterol and can subsequently cause the loss of cellular contents (Steel et al., 1988). In addition, induction of PCD by α-tomatine with hallmarks resembling apoptosis has been reported in the fungal tomato pathogen *Fusarium oxysporum* (Ito et al., 2007). Moreover, α-tomatine also possesses *in vitro* antibacterial activity despite the absence of sterols in the membrane of bacteria (Sparg et al., 2004). A recent study showed that tomato roots can secrete α-tomatine to modulate the rhizosphere microbial communities (Nakayasu et al., 2021).

Tomato fungal pathogens can detoxify α-tomatine by the activity of secreted glycosyl hydrolases (GH), referred to as “tomatinase”. So far, three types of tomatinase activities have been reported with hydrolysis of different sugar moieties and they can generate distinct breakdown products (You and van Kan, 2021).

This includes the removal of the lycotetraose and production of the aglycon tomatidine, such as in *Cladosporium fulvum* and *F. oxysporum* f. sp. *lycopersici* (Ökmen et al., 2013; Roldán-Arjona et al., 1999). Besides the entire lycotetraose, the terminal sugar moieties can also be hydrolysed by tomatinase, with β_2-tomatine as the breakdown product directly generated from the cleavage of the terminal glucose as described in *Septoria lycopersici* (Sandrock et al., 1995). A screening of *B. cinerea* strains revealed that most of them are capable of deglycosylating α-tomatine by removing the terminal D-xylose and generating β_1-tomatine (Quidde et al., 1998). Functional genetic studies showed that tomatinase activities also play a role in fungal virulence on tomato. Interestingly, the hydrolytic breakdown products, tomatidine and β_2-tomatine, were shown to suppress plant immunity and thereby serve as potential virulence-promoting factors (Bouarab et al., 2002; Ito et al., 2004).

Camalexin is the most thoroughly studied indole-derived phytoalexin in Arabidopsis. It is barely detectable in healthy Arabidopsis leaf tissues and is synthesized upon pathogen infection (Tsuji et al., 1992; Thomma et al., 1999; Mert-Turk et al., 2003) as well as in response to abiotic stresses such as UV light irradiation (Nawrath and Métraux, 1999). A series of QTLs controlling camalexin biosynthesis co-localized with QTLs that are associated with quantitative susceptibility to *B. cinerea* in Arabidopsis (Denby et al., 2004; Rowe and Kliebenstein, 2008). Moreover, the secretion of camalexin from the infected cells by the plasma membrane transporter proteins PEN3 and PDR12 has been reported to be required for resistance to *B. cinerea* in Arabidopsis (He et al., 2019).

B. cinerea isolates displayed different levels of sensitivity to camalexin *in vitro* and this trait is correlated with virulence on Arabidopsis. The camalexin-sensitive isolates formed larger lesions on camalexin-deficient Arabidopsis mutants than on the wild type leaves, whereas virulence of camalexin-tolerant isolates was not affected on Arabidopsis regardless of the absence or presence of camalexin (Kliebenstein et al., 2005). The gene encoding ATP-binding cassette (ABC) transporter *BcatrB* in *B. cinerea* was reported to be transcriptionally induced by camalexin and functions in the efflux of this toxic compound (Stefanato et al., 2009). Deletion of *BcatrB* rendered *B. cinerea* more sensitive to camalexin and less virulent on wild type Arabidopsis but did not impair the infection on Arabidopsis mutants defective in camalexin biosynthesis (Stefanato et al., 2009). Tomato does not synthesize camalexin, but it can produce a sesquiterpene compound named rishitin with *in vitro* antifungal activity. Rishitin was reported to rapidly accumulate in tomato leaves upon *B. cinerea* infection (Glazener et al., 1981), however, its role in defending tomato plants against *B. cinerea* in planta remains to be further elucidated (Charles et al., 2008).

Glucosinolates (GLs) and their derivatives (mainly breakdown products) exhibit *in vitro* toxicity against a broad range of pathogens (Buxdorf et al., 2013; Mari et al., 1993), and serve as phytoanticipins in

cruciferous plants. GLs can be degraded in response to biotic and abiotic stimuli by β-thioglucoside glucohydrolases also known as myrosinases; the toxic hydrolytic products, referred to as isothiocyanates (ITCs), play an important role in defense against pathogens and herbivores (Barth and Jander, 2006; Giamoustaris and Mithen, 1997; Poveda et al., 2020; Siemens and Mitchell-Olds, 1996). Arabidopsis mutant plants either compromised in GL biosynthesis (*myb28/myb29* and *cyp79b2/79b3*) or lacking the myrosinases to generate ITCs, exhibited increased susceptibility to fungal pathogens and insect herbivores (Barth and Jander, 2006; Buxdorf et al., 2013; Chen et al., 2020). On the other hand, *Brassica napus* transgenic plants overexpressing GL biosynthetic genes were more resistant against fungal pathogens than non-transgenic control plants (Zhang et al., 2015).

Recent studies have shed light on the detoxification mechanisms of GLs and ITCs in plant pathogens. Conversion of ITCs into less toxic products via the activity of a secreted hydrolase (ITCase) has been reported in the fungal pathogen *S. sclerotiorum* and contributed to its virulence on Arabidopsis (Chen et al., 2020). Besides, Major Facilitator Superfamily (MFS) transporter proteins can mediate the efflux of GL-breakdown products in *B. cinerea* (Vela-Corcía et al., 2019). A *B. cinerea* strain deficient in the MFS transporter BcmfsG was more sensitive to ITCs and less virulent on Arabidopsis plants containing GLs (Vela-Corcía et al., 2019).

Role of phytohormones in plant-*B. cinerea* interaction

Phytohormones and their corresponding signaling pathways are also involved in plant-*B. cinerea* interactions. In general, the positive of role of jasmonic acid (JA) in defense against necrotrophic pathogens including *B. cinerea* has been intensively studied and is well-acknowledged (Antico et al., 2012). For instance, tomato JA mutant *def1*, impaired in JA biosynthesis, has been reported to be more susceptible to *B. cinerea* (Díaz et al., 2002), while treatment of tomato fruits with MeJA or the activation of JA biosynthesis by alkamides in Arabidopsis increased resistance to *B. cinerea* (Shu et al., 2020; Mendez-Bravo et al., 2011; Yu et al., 2009).

On the contrary, salicylic acid (SA) is well-recognized to contribute to the defense against biotrophic pathogens (Díaz et al., 2002; Glazebrook, 2005). Application of SA or its analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) on Arabidopsis and tomato conferred increased resistance to *B. cinerea* (Audenaert et al., 2002; Zimmerli et al., 2001). Also, it has been reported that *B. cinerea* produces an exopolysaccharide that activates the SA signaling pathway which consequently promotes infection on Arabidopsis probably through antagonism of the JA pathway (El Oirdi et al., 2011). However, the exact role of SA-dependent signaling pathways in resistance to the necrotrophic pathogen *B. cinerea* is still

ambiguous. On the one hand, tomato plants with decreased SA accumulation resulting from overexpression of a bacterial SA hydroxylase (NahG), were shown to be more susceptible to *B. cinerea* (Audenaert et al., 2002; El Oirdi et al., 2011). On the other hand, *Nicotiana benthamiana* transgenic plants (NahG) with decreased SA accumulation did not show significant difference in susceptibility to *B. cinerea* (Asai et al., 2010). Arabidopsis plants transformed with NahG exhibited contradictory outcomes in disease resistance. Thomma *et al*. (1998) first reported that Arabidopsis NahG-overexpressing transgenic plants were not affected in susceptibility to *B. cinerea*, an observation that was corroborated by Veronese *et al*. (2004) and Ferrari *et al*. (2003) and several Arabidopsis mutants either defective in SA biosynthesis or perception did not show enhanced disease symptoms (Ferrari et al., 2003). However, other studies reported that expression of *NahG* in Arabidopsis can reduce the resistance to *B. cinerea* (Govrin and Levine, 2002; Zimmerli et al., 2001).

Abscisic acid (ABA) is generally believed to promote susceptibility to *B. cinerea* (Mbengue et al., 2016). The exogenous application of ABA on tomato leaf or increasing ABA content in Arabidopsis led to a higher level of susceptibility to *B. cinerea* (Audenaert et al., 2002; Liu et al., 2015). Impairment of ABA biosynthesis in tomato and Arabidopsis resulted in elevated resistance to *B. cinerea* (Audenaert et al., 2002; Korolev et al., 2008).The resistance mechanism conferred by ABA deficiency is related to the increase in permeability of the cuticle and, as a consequence, the early perception of fungal attack and timely activation of defense responses (Asselbergh et al., 2007; Curvers et al., 2010; Sivakumaran et al., 2016). Manipulation of the host ABA response by *B. cinerea* has also been reported. The fungus was observed to synthesize ABA or induce host ABA production to gain advantage during infection (Siewers et al.,2004; Siewers et al., 2006).

Ethylene (ET) is required for resistance against *B. cinerea*. Tomato plants pretreated with ET showed increased expression of pathogenesis-related genes and exhibited decreased susceptibility, whereas pretreatment with ET perception inhibitors or mutations in the ET signaling pathway attenuated resistance towards *B. cinerea* (Berrocal-Lobo et al., 2002; Díaz et al., 2002).

Transcriptome analysis as an important tool for studying plant-*B. cinerea* interactions

Transcriptome analysis based on IIIumina high-throughput sequencing has been widely used to study the infection strategies of *B. cinerea* and the defense responses on a genome-wide scale in hosts including Arabidopsis, tomato, strawberry, grape, rose, cucumber, etc. (**Table 1**) Through comparative transcriptome analysis, several important characteristics in plant-*B. cinerea* interactions have been addressed. In general, the plant response to *B. cinerea* infection involves large-scale transcriptional

reprogramming at an early time point. In Arabidopsis, around one-third of the genes in the genome was differentially expressed in the first 48 hours (hrs) after inoculation with *B. cinerea* (Windram et al., 2012). More than 2000 strawberry genes were differentially expressed at 24 hrs post inoculation in fruits (Haile et al., 2019). In addition, resistance mechanisms revealed by transcriptome analysis are associated with complex gene regulatory networks. Transcription factors and phytohormone signaling pathways were significantly enriched in the differentially expressed genes (DEG) in response to *B. cinerea* infection (Blanco-Ulate et al., 2013; Liu et al., 2018; Zhang et al., 2017). Besides, a comparative transcriptome study of resistance mechanisms in the wild tomato relative, *S. lycopersicoides* revealed that a prompt plant response to *B. cinerea* comprises the suppression of processes involved in growth and photosynthesis and simultaneous induction of defense-related genes, including pathogenesis-related proteins (Smith et al., 2014). However, a recent study attributed the fact that ripe tomato fruits become susceptible to *B. cinerea* mainly to the increase in expression of genes promoting susceptibility rather than to a decrease in defense responses (Silva et al., 2021).

Virulence mechanisms employed by *B. cinerea* were also explored by RNA-seq based transcriptome studies. Induction of *B. cinerea* genes encoding secreted effectors, biosynthesis of toxins, enzymes involved in plant cell wall degradation and autophagy-related proteins was observed indicating that successful infection might require the coordination of different infection strategies (Reboledo et al., 2021; Zhang et al., 2020). Moreover, a transcriptome study of the *B. cinerea* germination process on artificial surface mimicking the host plant cuticle uncovered the priming of host invasion-related genes before penetration (Leroch et al., 2013). Similarly, RNA-seq analysis of ascospores resulting from sexual reproduction in *B. cinerea* revealed increased transcript levels of host invasion-related genes indicating that the ascospores are transcriptionally primed for infection before being released from apothecia (Rodenburg et al., 2018).

CRISPR (clustered regularly interspaced short palindromic repeats) together with Cas9 (CRISPR-associated protein 9) constitutes one form of bacterial acquired immunity (Barrangou et al., 2007; Gasiunas et al., 2012; Wiedenheft et al., 2012). The discovery of the CRISPR/Cas9 system and subsequent establishment and application of genome editing technology have revolutionized molecular biology studies and was awarded the Nobel Prize in 2020. In brief, a genomic sequence can be specifically recognized by a guide RNA, the Cas9 protein can then cause double-strand breaks (DSBs) in the target DNA sequences upstream of the protospacer adjacent motif (PAM). The DSBs are then repaired by either of two well-characterized mechanisms, non-homologous end joining (NHEJ) or homologous recombination (HR). The NHEJ pathway mostly causes random mutations such as insertions or deletions (indel) causing a frame-shift (resulting in

functional knock-out), whereas DSBs repaired by HR can replace the target sequence by a selection marker (knock-out), by an altered version of the target sequence (gene editing) or by a sequence carrying an in-frame reporter gene construct (knock-in) (Bortesi and Fischer, 2015). A recent study using an intron-optimized Cas9 gene proved the ability of efficient editing of multiple targets (8 genes in *N. benthamiana* and 12 genes in Arabidopsis) in one single transformation event (Stuttmann et al., 2021).

Table 1. RNA-seq based transcriptome analyses of plants inoculated with *B. cinerea*

Host plant	Tissue	Reference
Arabidopsis (*Arabidopsis thaliana*)	leaf	(Windram et al., 2012)
Arabidopsis (*Arabidopsis thaliana*)	leaf	(Zhang et al., 2017)
Lettuce (*Lactuca sativa*)	leaf	(De Cremer et al., 2013)
Cultivated tomato (*Solanum lycopersicum*)	fruit	(Blanco-Ulate et al., 2013)
Cultivated tomato (*Solanum lycopersicum*)	leaf	(Zhang et al., 2020)
Cultivated tomato (*Solanum lycopersicum*)	leaf/fruit	(Vega et al., 2015)
Cultivated tomato (*Solanum lycopersicum*)	fruit	(Silva et al., 2021)
Cultivated tomato (*Solanum lycopersicum*)	leaf	(Srivastava et al., 2020)
Wild tomato (*Solanum lycopersicoides*)	leaf	(Smith et al., 2014)
Rose (*Rosa* sp.)	petal	(Liu et al., 2018)
Strawberry (*Fragaria ananassa*)	fruit	(Xiong et al., 2018)
Wild strawberry (*Fragaria vesca*)	fruit	(Haile et al., 2019)
Strawberry (*Fragaria ananassa*)	fruit	(Lee et al., 2021)
Kiwi (*Actinidia chinensis* var.*deliciosa*)	fruit	(Zambounis et al., 2021)
Grape (*Vitis vinifera*)	berry	(Zhang et al., 2020)
Grape (*Vitis vinifera*)	berry	(Agudelo-Romero et al., 2020)
Grape (*Vitis vinifera*)	berry	(Haile et al., 2020)
Wild grape (*Vitis amurensis*)	leaf	(Wan et al., 2021)
Moss (*Bryophyte Physcomitrium*)	whole tissue	(Reboledo et al., 2020)
Cucumber (*Cucumis sativa*)	leaf	(Kong et al., 2015)

Until now, the CRISPR/Cas9-mediated mutagenesis has been successfully applied to 45 plant genera across 24 families including both genetic model species and agriculturally important plants (Shan et al., 2020; Zhu et al., 2020). The first tomato mutant generated by the CRISPR/Cas9 system was reported in 2014 (Brooks et al., 2014) and the method has been widely used to study the mechanisms underlying disease resistance in tomato (**Table 2**). Knockout of tomato genes encoding proteins involved in immunity including mitogen-activated protein kinases (MAPKs) or Non-expressor of Pathogenesis-Related gene 1 (NPR1) compromised resistance to *B. cinerea* (Li et al., 2020; Zhang et al., 2018). On the contrary, knockout of a tomato pectate lyase (PL) gene that acts as a susceptibility factor improved fruit resistance to *B. cinerea* (Silva et al., 2021). The CRISPR/Cas9 genome editing technology was also developed in microbes and has been established in more than 40 different species of filamentous fungi and oomycetes including plant pathogens such as *B. cinerea* (Leisen et al., 2022) and *Phytophthora infestans* (Schuster and Kahmann, 2019). The application of

CRISPR/Cas9 in fungi involved different Cas9 and sgRNA delivery strategies. Transgenic expression of a codon-optimized Cas9 gene and sgRNAs under the control of strong fungal promoters can take place in transformed fungal cells via chromosomal integration or an autonomously replicating plasmid. Another method is to use the purified Cas9 protein and *in vitro* synthesized sgRNAs to form a Cas9-sgRNA ribonucleoprotein (RNP) complex and transform the fungal protoplasts with RNP complex mediated by polyethylene glycol (PEG). One of the major advantages of the CRISPR/Cas9 system is that the repair of DBS via HR requires short homologous overhang (Song et al., 2019). According to Leisen *et al*. (2020), 60 bp flanks at the borders of DSBs is sufficient for high incidence of target-specific integration (>90%), whereas the conventional *B. cinerea* transformation protocol described by Hamada *et al*. (1994) requires 0.5 to 1 kb homologous flanks. Because of the limited half-life of RNP complexes in fungal cells, the CRISPR/Cas9 system allows precise genome editing with low incidence of off-target mutations as revealed by genome sequencing of the transformants (Leisen et al., 2020). Moreover, multiple mutagenesis sites can be targeted simultaneously by adding several sgRNAs during the transformation, for instance, double mutants can be generated in one transformation assay simply through using two sgRNAs and two different selection markers (Hahn and Scalliet, 2021). Besides, a recent study optimized the CRISPR/Cas9 system and enabled marker-free serial deletion of 12 genes in *B. cinerea* (Leisen et al., 2022)

In summary, the application of the CRISPR/Cas9 system to plants and microbes enables flexible and efficient genome editing and has greatly revolutionized studies on gene function and with further exploitation will continue to promote our understanding of plant-microbe interactions.

Table 2. Mutations in tomato genes generated by CRISPR/Cas9 system to study plant-microbe interactions

Target	Type of Pathogen	Causal agent	Reference
SlMYC2	Fungus	*B. cinerea*	(Shu et al., 2020)
SlMAPK3	Fungus	*B. cinerea*	(Zhang et al., 2018)
SlNPR1	Fungus	*B. cinerea*	(Wang et al., 2020)
SlPL	Fungus	*B.* cinerea	(Silva et al., 2021)
SlPMR4	Fungus	*Oidium neolycopersici*	(Martínez et al., 2020)
miR482b and miR482c	Oomycete	*Phytophthora infestans*	(Hong et al., 2021)
SlJAZ2	Bacterium	*Pseudomonas syringae* pv. *tomato (Pto)* DC3000	(Ortigosa et al., 2019)
SlPelo and SlMlo1	Virus and fungus	Yellow Leaf Curl Virus and *Oidium neolycopersici*	(Pramanik et al., 2021)
SleIF4E1	Virus	Pepper Mottle Virus	(Yoon et al., 2020)
SlCCD8	Parasite	*Phelipanche aegyptiaca* and *Orobanche* spp.	(Bari et al., 2019)
SlMAX1	Parasite	*Phelipanche aegyptiaca*	(Bari et al., 2021)

Research questions

The main objective of this PhD project was to study the interaction of *B. cinerea* and tomato. To this end, I addressed several scientific questions:

1. What are the resistance mechanisms of wild tomato *S. habrochaites* LYC4 against *B. cinerea* in comparison with *S. lycopersicum* MM?
2. Which gene encodes the tomatinase in *B. cinerea* isolate B05.10?
3. What is the basis of tomatinase-deficiency and reduced virulence on tomato of *B. cinerea* isolate M3a ?
4. Are there non-hydrolytic tolerance mechanisms to α-tomatine in *B. cinerea*?
5. How important is tolerance to α-tomatine for virulence of *B. cinerea* on tomato?
6. How important is α-tomatine for basal resistance of tomato to *B. cinerea* and other pathogens?

Thesis outline

In Chapter II, we tested the partial resistance to *B. cinerea* in LYC4 as well as its introgression lines that were earlier described using different inoculation methods. We describe inoculation conditions that can reproducibly and predictably achieve a distinct infection outcome (compatible interaction vs incompatible interaction) between LYC4 and MM.

In Chapter III, we further investigate the mechanisms of compatibility and incompatibility in the interactions between *B. cinerea* and tomato using *S. habrochaites* LYC4 and *S. lycopersicum* MM and different inoculation conditions. RNA-seq analysis was employed to studied the transcriptional changes in both *B. cinerea* and the host plants at early time points during fungal infection.

Chapter IV reviews the state of knowledge about the role of α-tomatine in plant-microbe interactions, before the start of this thesis project. We discuss several findings which extend beyond the simple perception that plants produce membrane-perforating toxins which pathogens can inactivate by hydrolysis. We also address a number of outstanding questions that deserve attention in future research

Chapter V describes the transcriptional response to α-tomatine treatment in B05.10 aiming to identify the hydrolytic and non- hydrolytic tolerance mechanisms to α-tomatine, and we sequenced the genome of *B. cinerea* isolate M3a to reveal the molecular basis of tomatinase deficiency. Functional analyses of α-tomatine-responsive genes and their encode proteins were performed to unravel their contribution to the tolerance of *B. cinerea* to α-tomatine.

Although α-tomatine is recognized as a defense compound that can protect tomato plants from pathogens and herbivores, direct functional evidence about the importance of α-tomatine from transgenic tomato plants with altered α-tomatine levels (either reduced or increased) is still missing. In **Chapter VI**, we describe the deletion of two α-tomatine biosynthetic genes *GAME2* and *GAME4* by CRISPR-Cas9 and we overexpressed these two genes separately in MM to investigate the role of α-tomatine in basal resistance. Finally, the most important findings of this thesis project are discussed in **Chapter VII**.

Chapter 2

Stem and leaf resistance to *Botrytis cinerea* in wild tomato *Solanum habrochaites* LYC4

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Abstract

Tomato (S*olanum lycopersicum*) cv. Moneymaker (MM) is very susceptible to the grey mould *Botrytis cinerea,* while quantitative resistance in the wild species *Solanum habrochaites* (accession LYC4) has been reported. We evaluated stem and leaf resistance to *B. cinerea* in LYC4 and eight introgression lines each containing distinct genome segments of LYC4 in the MM background. For this purpose, we used a new stem infection procedure by inoculating whole plants with *B. cinerea* conidia suspensions on petiole stubs generated by leaf pruning. A high level of stem resistance was observed in LYC4, whereas the introgression lines showed intermediate levels of resistance as compared with MM. In leaf infection assays, an effect of nutrient and spore concentration in disease incidence was observed. Resistance in LYC4 leaves was most pronounced when *B. cinerea* was inoculated at high spore density (1000 spores/µL) in medium with low sugar (10 mM sucrose) and manifested as a high incidence of tiny black, dispersed spots which did not expand (incompatible interaction). Under the same condition, a high frequency of expanding lesions was observed when inoculating MM leaves (compatible interaction). Remarkably, leaf resistance in LYC4 was abolished when inoculation was performed either with low spore density (30 spores/µL) or with inoculum at high spore density supplemented with 50 mM sucrose, leading to a high percentage of expanding lesions. Histochemical staining of reactive oxygen species (ROS) and cell wall fortification at early time points (within 24 hours) revealed an earlier and more intense staining during a compatible interaction in MM than in the incompatible interaction in LYC4.

Introduction

The grey mould fungus, *Botrytis cinerea* is a necrotrophic pathogenic fungus with a wide host range. It can infect >1400 plant species (Elad et al., 2016), including many dozens of important food crops and ornamental plants, mainly in temperate climate regions. Among the cash crops affected most by grey mould are rose, gerbera, tomato, grapevine and strawberry. The damage that grey mould inflicts worldwide to producers, traders and consumers exceeds €2 x 10^9 per year (Dean et al., 2012).

Quantitative resistance to *B. cinerea* was identified in several wild relatives of tomato (*Solanum lycopersicum*), including *Solanum habrochaites* LYC4 (ten Have et al., 2007). An interspecific cross between *S. lycopersicum* Moneymaker (MM) and *S. habrochaites* LYC4 was made and the genetics of resistance to *B. cinerea* was studied in an F2 population (Finkers et al., 2007a). Subsequently, an introgression line (IL) population was constructed consisting of 30 unique genotypes containing homozygous introgressions of segments of the LYC4 genome in the susceptible cultivated tomato background (Finkers et al., 2007b). Several ILs showed increased resistance to *B. cinerea*. A total of ten quantitative trait loci (QTLs), located on 8 chromosomes, were identified in the IL population contributing to partial resistance to *B. cinerea* (Finkers et al., 2007a,b). Six of the ten QTLs conferred a reduction of disease incidence, i.e. the proportion of inoculation points leading to expanding lesions, while four QTLs conferred a slower lesion growth rate (in mm/day). These disease parameters are considered to be, at least partially, governed by different genetic traits and may involve different underlying physiological mechanisms. QTLs on chromosomes 1 and 4, as well as those on chromosomes 9 and 12 have an additive mode of action. One genotype possessing two QTLs displayed a reduction in disease incidence of 80% and a reduction in lesion growth rate of 40%, as compared to the susceptible control (Finkers et al., 2007b). These results indicate that combining multiple resistance mechanisms may lead to enhanced resistance.

B. cinerea inoculation under laboratory conditions, as commonly performed to study its interaction with host plants, requires a carbon source and phosphate in the inoculum to enable infection (van Den Heuvel, 1981). Gamborg's B5 (GB5) supplemented with sucrose or glucose and potassium phosphate has been used to establish consistent infection on detached tomato leaves under lab conditions (Benito et al., 1998). When inoculating MM leaves, serving as a susceptible control, 10 mM sucrose and 10 mM potassium phosphate is sufficient to cause synchronized and reproducible occurrence of expanding lesions (Benito et al., 1998). By contrast, the same inoculation conditions on wild tomato, such as *S. lycopersicoides*, most frequently resulted in non-expanding lesions which displayed as small black spots under the inoculation droplet (Guimarães et al., 2004). Besides, conidia applied on the leaves of a tomato abscisic acid (ABA)-

deficient mutant with 10 mM glucose also barely developed expanding lesions but rather triggered the formation of black spots that did not expand. However, the frequency of brownish expanding lesions increased either by the addition of potassium phosphate or by providing higher concentrations of glucose in the inoculum (Audenaert et al., 2002). How the addition of sugars or phosphates can facilitate *B. cinerea* infection on tomato leaves is not well understood.

Except for the effect of nutrient supplements, the impact of spore density in the inoculum on the infection outcome has not been experimentally studied in *B. cinerea*. In most published experiments, densities ranging from 100 to 1000 spores per µL were used for *B. cinerea* inoculation. It has been proposed that the initial spore density of *B. cinerea* infection might determine the amplitude of fungal attack on the host and the subsequent plant defense responses (Veloso and van Kan, 2018). The fungal pathogen *Plectosphaerella cucumerina* has been reported to display either hemibiotrophic or necrotrophic behaviour, depending on the initial spore densities (Pétriacq et al., 2016). Inoculation with high spore densities induced Jasmonic acid-dependent defense responses indicating the infection is recognized as attack of necrotrophic pathogen. In comparison, low spore density inoculation induced salicylic acid-mediated defenses which are generally activated by hemibiotrophic pathogens (Pétriacq et al., 2016). Also for *B. cinerea*, it should be considered that the initial spore densities in the inoculum might play an important role in infection strategies and affect plant defenses in a complex way.

The oxidative burst which is characterized by rapid production of ROS, including hydrogen peroxide (H_2O_2) plays an important role during host-microbe interactions. It is a key regulator in defense responses because it activates the expression of defense-related genes, cell wall fortification and the host programmed cell death response. Besides, ROS also are toxic to microbes and *B. cinerea* can cope with the oxidative stress by secreting enzymes functioning in ROS scavenging including catalases and peroxidases during plant infection (Siegmund and Viefhues, 2016). However, as a necrotrophic pathogen, *B. cinerea* generally benefits from a host cell death response to enable lesion growth and therefore, ROS production has been suggested to assist *B. cinerea* infection (Elad, 1992; Govrin and Levine, 2000). Suppression of plant-generated ROS during *B. cinerea* infection through infiltration of diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase involved in ROS production in *Arabidopsis* leaves, significantly confined lesion expansion (Govrin and Levine, 2000). On the other hand, enhancement of ROS generation in plant tissues resulting from infiltration of mixtures either containing xanthine oxidase plus xanthine or of glucose oxidase plus glucose, gave rise to larger lesions upon *B. cinerea* inoculation (Govrin and Levine, 2000). However, the view that ROS generation is by definition beneficial for *B. cinerea* was refuted by a study from Asselbergh et al. (2007). Upon inoculation on a *S. lycopersicum* ABA-deficient mutant named "*sitiens*"

with high levels of resistance to *B. cinerea*, earlier and stronger ROS production coupled with cell wall fortification and protein cross-linking was observed in the epidermal cells. Moreover, application of ascorbate or DPI impaired the ROS burst and compromised the resistance in *sitiens* (Asselbergh et al., 2007). Based on the existing evidence from different infection systems, it seems that a ROS burst can play a dual role. On the one hand, induction of a ROS burst is required for host cell death and thereby promotes lesion expansion especially at later stages, while on the other hand, the timely and abundant production of ROS and the subsequent plant defenses in the early stage can efficiently block the fungal invasion. Above all, these observations indicate that the exact role of the host plant ROS burst in resistance against *B. cinerea* needs to be further studied.

Results

Evaluation of resistance to *B. cinerea* of the introgression lines with nutrient-rich inoculum

To confirm the resistance against *B. cinerea* in the introgression lines generated from MM and its wild relative LYC4 (Finkers et al., 2007), eight introgression lines (ILs) as well as the parental lines MM and LYC4 were inoculated with conidia suspensions in PDB medium on stems and leaves, respectively. To assess the resistance level, we recorded two parameters: the proportion of inoculation droplets that caused expanding lesions (disease incidence) and the diameter or area of expanding lesions (lesion size). The experimental setup for the stem inoculation is illustrated in **Figure 1A-B** and some typical symptoms observed are illustrated in **Figure 1C-K**. Petiole stub inoculations on LYC4 or resistant ILs resulted in necrotic symptoms that remained confined to the petioles or nodes, and did not cause severe symptoms on or inside the stem (**Figure 1D-F**). By contrast, inoculations on MM petiole stubs mostly developed into lesions that expanded up or down the stem periphery and eventually girdled the stem (**Figure 1G-H**). Longitudinal sections of stem segments were performed to check the correlation between external symptoms and internal damage in the stem tissue. Stem damage in partially resistant genotypes was restricted to the node surface (**Figure 1I**) or was superficially spreading along the stem (**Figure 1J**), while in susceptible genotypes the fungus invaded and destroyed the core of the stem (**Figure 1K**). Eventually, infections on MM resulted in collapse of the entire plant (**Figure 1C**).

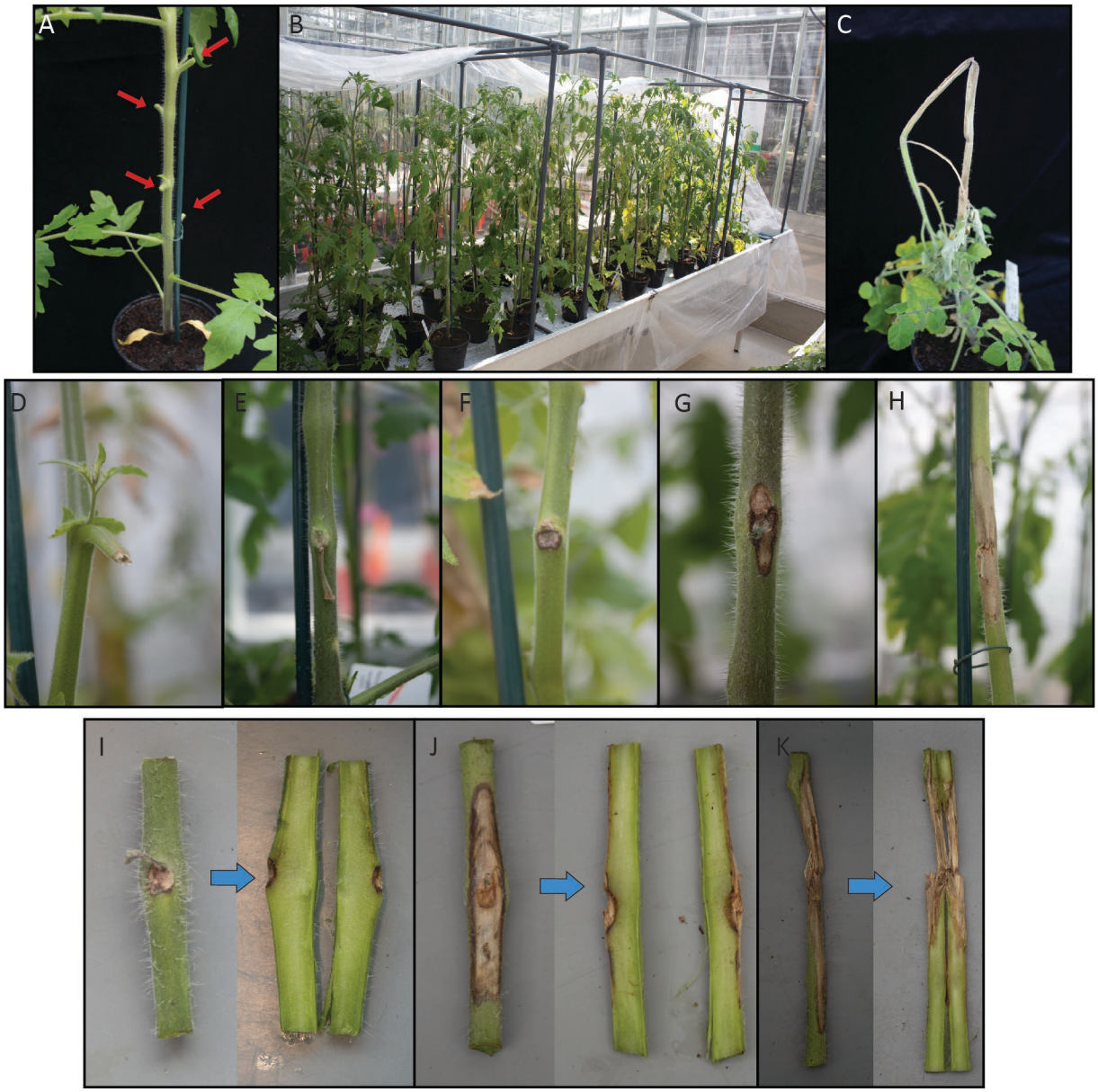

Figure 1. Symptoms of *B. cinerea* infection following petiole stub inoculation. Petiole stubs that were inoculated in assays on whole plants are marked by red arrows (A). Stem assay in greenhouse, plants were put in the frame covered with plastic sheets for the first two days under high humidity (B). Collapse of tomato plant resulting from stem infection (C). Incompatible interaction; fungal infection restricted to the petiole stump, which fully collapses (D). Incompatible interaction; fungal infection caused shrinkage or abscission of the petiole but did not reach the stem (E). Incompatible interaction; fungal infection only colonized the surface of the node but did not progress into the stem (F). Compatible interaction; disease progressed slowly beyond the node over the stem surface and internally, the stem lesion displayed dark brown edges (G). Compatible interaction; fast spreading lesion, lesion girdled the entire stem eventually leading to plant collapse (H). Longitudinal section of incompatible interaction; fungal infection only leads to discoloration of the node surface (I). Longitudinal section of slowly progressing lesion; fungal infection mainly colonizes the stem surface (J). Longitudinal section of rotten stem; full colonization of the stem tissue, causing a hollow stem (K).

In leaf assays, eight selected ILs were tested alongside their parent genotypes, MM and LYC4. The genotypes IL 1-3/3-3, IL 4-1 and LYC4 displayed significantly smaller average lesion sizes than MM, however, IL 2-1, IL 9-1, IL 9-2 and IL 11-2 were more susceptible to *B. cinerea* than MM as manifested by larger average lesion sizes (**Figure 2A**). None of the genotypes, including LYC4, displayed significantly lower disease incidence than MM in leaf inoculation assays (**Figure 2B**). Stem resistance was confirmed in six of the eight ILs, except IL 2-1 and IL 9-2. The average lesion size of IL 2-1 was significantly larger than MM, whereas the average lesion size of IL 9-2 did not exhibit difference to MM (**Figure 2C**). Most of the inoculum droplets on leaves formed primary lesions in the first 24 hpi and expanded afterwards. The disease incidence did not significantly differ between the ILs and MM (**Figure 2D**).

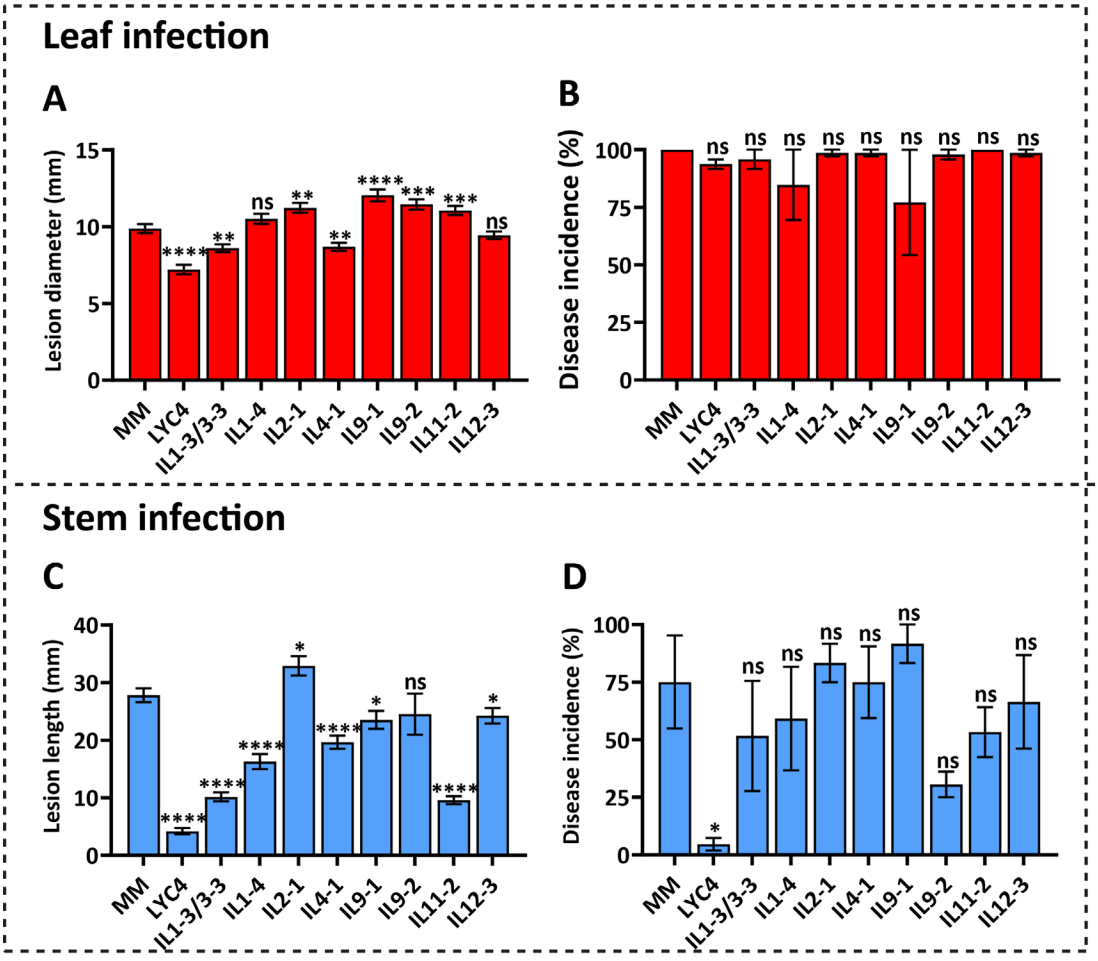

Figure 2. Disease parameters of leaf and stem infection assays on the ILs, LYC4 and MM. Percentage of expanding lesions from stem infection assays recorded at 6 dpi (A); Lesion length of expanding lesions from stem infection assays recorded at 6 dpi (B); Percentage of expanding lesions from leaf infection assays recorded at 2 dpi (C); Lesion diameters of expanding lesions from leaf infection assays recorded at 2 dpi (D); Data are displayed as mean values ± standard error of mean (SEM) of all measurements from three independent infection assays. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value <0.01; *** p-value <0.005; **** p-value <0.0001). ns indicates no significant difference.

Exploration of the incompatible interaction between *B. cinerea* and LYC4

Because *B. cinerea* inoculation typically requires nutrient supply in the inoculum to initiate infection, we prepared the spore inoculum in potato dextrose broth (PDB), which is commonly used in *B. cinerea* infection. Most of the leaf inoculations on LYC4 caused expanding lesions even though the lesions were consistently smaller than on MM. We considered that the strong infection promoting effect of PDB, because of its abundance in nutrients, might minimize the detectable difference in resistance against *B. cinerea* between LYC4 and MM. Thus, we used a synthetic medium composed of Gamborg's B5 (GB5) salts, sucrose and phosphate which allows to specifically control the concentration of different components to manipulate the aggressiveness of *B. cinerea* during artificial infection.

In contrast to the high incidence of expanding lesions (referred to as compatible interaction) from PDB infection, spore suspensions in GB5 supplemented with 10 mM sucrose and 10 mM potassium phosphate, referred to as GB5-10 mM sucrose-10 mM phosphate hereafter, caused a higher proportion of non-expanding lesions, visible as dark spots or in some cases scattered black dots mainly restricted to the area of the inoculation droplet (**Figure 3A**). Most of these primary lesions did not expand even after prolonged incubation. The same inoculation conditions on MM leaves resulted in a high percentage of expanding lesions (**Figure 3C**). When the sucrose concentration in the inoculum was increased from 10 mM to 50 mM, the proportion of spreading/expanding lesions that were formed on LYC4 increased. On MM the proportion of expanding lesions at 10 mM was already high, however, the increase of sucrose to 50 mM resulted in a significant increase in the lesion size at 3 dpi (**Figure 3B**).

We analyzed the effect of initial spore densities (30 spores/µL, 100 spores/µL, 300 spores/µL and 1000 spores/µL) on the outcome of *B. cinerea* infection on LYC4. When the inoculum containing 10 mM sucrose was used, 1000 spores/µL exhibited low disease incidence on LYC4. Reducing the spore density to 30 or 100/µL significantly increased the proportion of expanding lesions to ~50% (**Figure 3D**).

Most of the inoculations on LYC4 could form expanding lesions when inoculum was supplemented with 50 mM sucrose, but lower spore densities (30 spores/µL and 100 spores/µL) resulted in substantially smaller lesions than inoculation with high spore densities (300 spores/µL and 1000 spores/µL) (**Figure 4**).

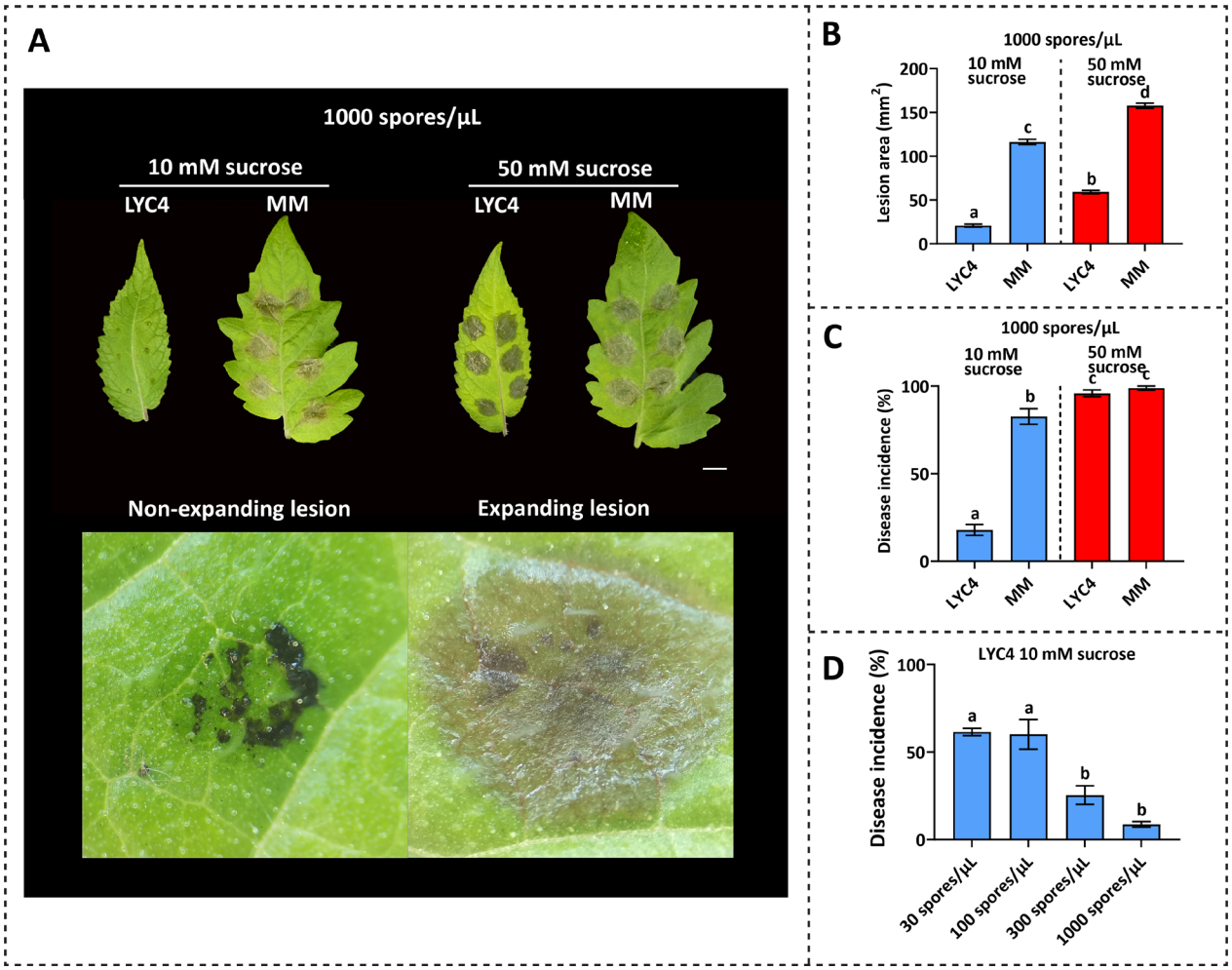

Figure 3. Effect of sugar concentration and spore density on *B. cinerea* infection on MM and LYC4. Detached leaves were inoculated with 2 µL inoculum containing 1000 spores/µL. Symptoms were recorded at 3 dpi. Infection symptoms of *B. cinerea* inoculation supplemented with different sucrose concentration in the inoculum (A); Binocular image of non-expanding and expanding on LYC4 (B); Disease incidence upon inoculation with GB5 medium containing different sucrose concentrations on LYC4 and MM (C); Lesion area of expanding lesions from inoculation with different sucrose concentrations on LYC4 and MM (D); Disease incidence upon inoculation with different spore densities using GB5-10 mM sucrose-10 mM phosphate medium on LYC4 (E); Data are displayed as mean values ± SEM of all measurements from at least three independent infection assays. Different letters indicate significant difference. Scale bar indicates 1 cm.

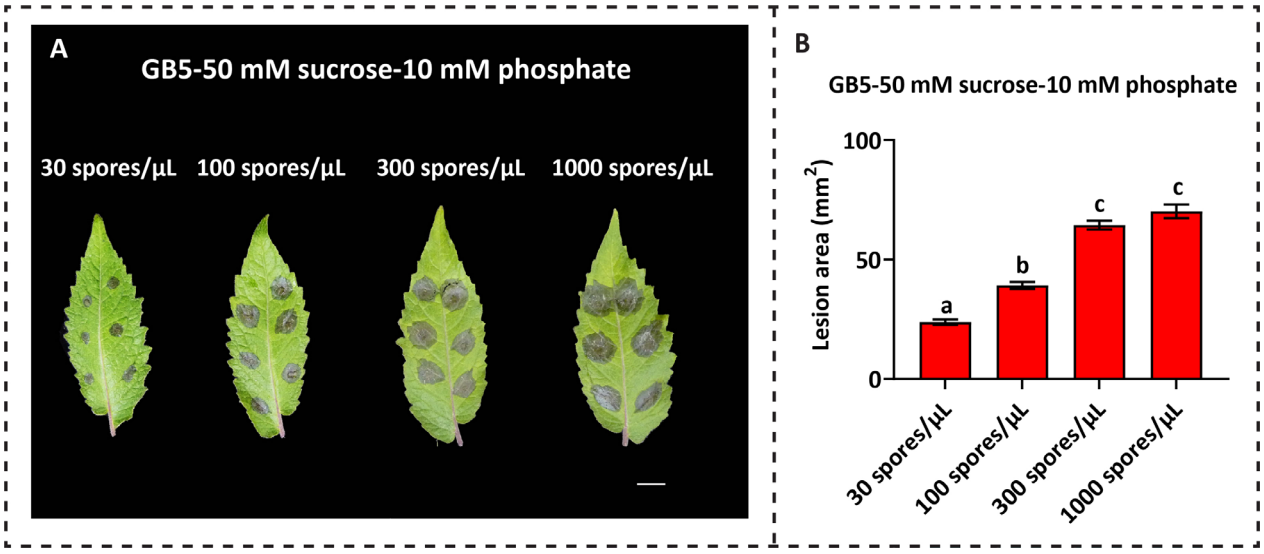

Figure 4. *B. cinerea* inoculation with different spore densities on LYC4 leaves in 50 mM sucrose. Symptoms were recorded at 3 dpi. Infection symptoms of *B. cinerea* inoculation with different spore densities on LYC4 (A). Lesion area of *B. cinerea* inoculation with different spore densities on LYC4 at 3 dpi (B). Data are displayed as mean values ± SEM of all measurements from at least three independent infection assays. Different letters indicate significant difference. Scale bar indicates 1 cm.

Histochemical analysis of plant responses during compatible and incompatible interactions

We analyzed the cell wall enforcement and H_2O_2 accumulation during *B. cinerea* infection on LYC4 and MM. It has been shown that resistance to *B. cinerea* in the *S. lycopersicum* mutant *sitiens* was accompanied by earlier and stronger production of H_2O_2 and cell wall modifications (Asselbergh et al., 2007). On the contrary, we observed that H_2O_2 accumulation in LYC4 was more dispersed and occurred in fewer epidermal cells than in MM (**Figure 5A**) Moreover, lignin deposition revealed by toluidine blue (TB) staining was delayed on LYC4 as compared to MM (Figure 5 B). These preliminary observations suggested a novel pattern of defence responses involved in resistance in LYC4, which require further investigations to better understand the resistance mechanism.

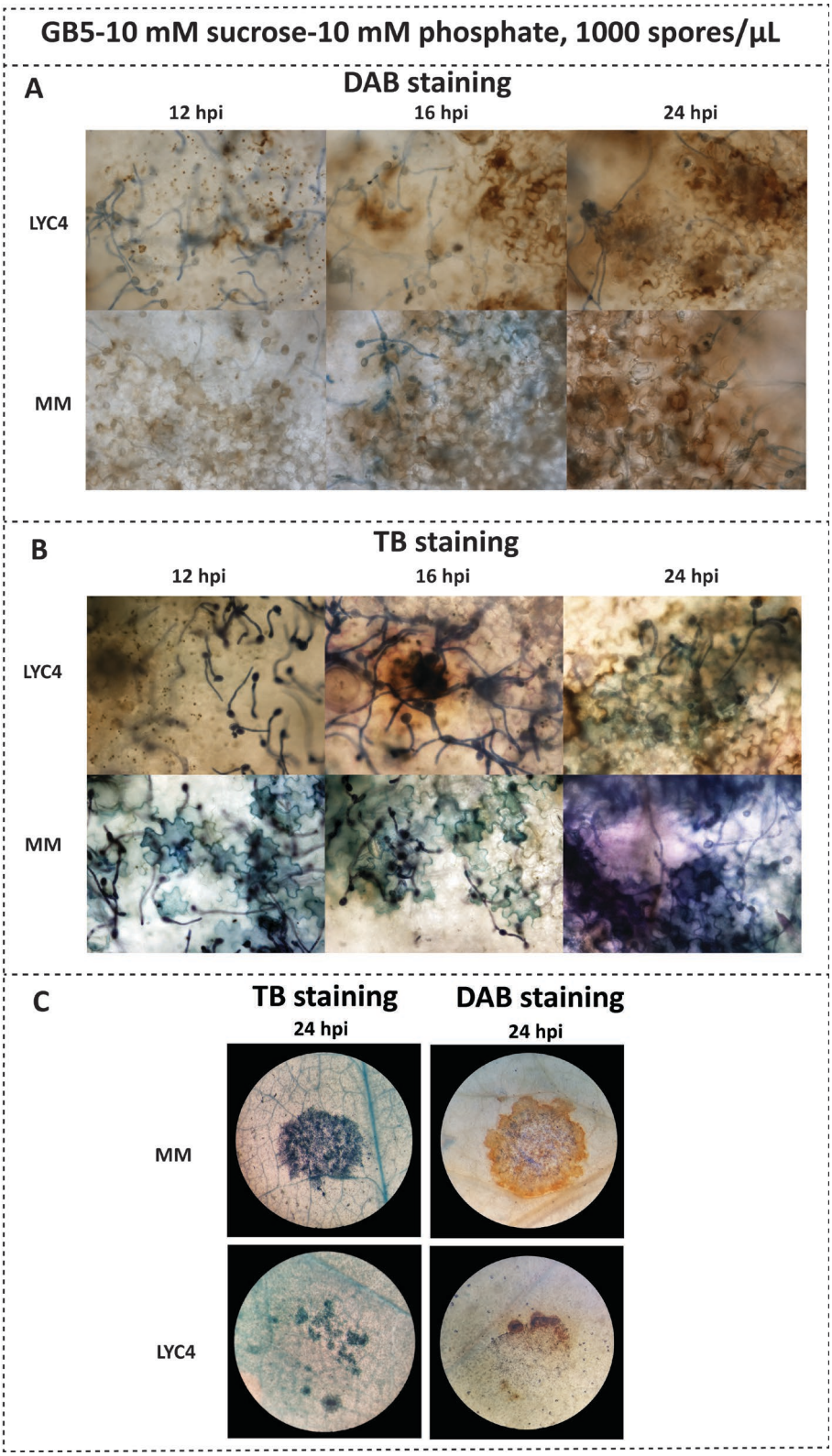

Figure 5. Histochemical staining of *B. cinerea* infection on MM and LYC4 leaves using GB5 10 mM sucrose medium containing 1000 spores/µL as inoculum. H_2O_2 accumulation on MM and LYC4 leaves after inoculation with *B. cinerea* manifested by 3, 3'-diaminobenzidine (DAB) staining (A). Cell wall modifications on MM and LYC4 leaves inoculated with *B. cinerea* manifested by Toluidine Blue (TB) staining (B). Binocular images of DAB and TB staining on LYC4 at 24 hpi (C).

Discussion

Partial resistance to *B. cinerea* in LYC4 and several introgression lines was assessed in this study in both leaf and stem assays. We developed a new stem infection method through pipetting spore suspensions in PDB on the wounded sites of petiole stubs after leaf detachment from intact 5-week-old plants. This procedure is much easier compared with the stem infection method used by ten Have et al. (2007), which required cutting the stem in segments and inoculating the segments on the top, or the method used by Finkers et al. (2007) which required large green-house space and inoculation into incision wounds on the stem and taping the stems to seal the wound and create high humidity. This method generally enabled more reproducible infections than the previous methods on up to three petiole stubs per plant. *B. cinerea* inoculation on LYC4 petiole stubs barely caused expanding lesions on the stems, whereas most inoculations on MM resulted in fungal growth into the central stem tissues and eventually to collapse of the whole plant. The selected introgression lines harboring different resistance QTLs displayed high incidence of spreading lesions on stems but with smaller lesions than MM. It is worth mentioning that the stems of LYC4 are very woody and clearly have a very different content of cell wall polymers, whereas all introgression lines have a much more normal stem tissue appearance alike MM. Possibly, the stem resistance in LYC4 is not necessarily related to defense but at least partially due to the very unattractive substrate that LYC4 stem tissue provides (as compared to MM). It is known that *B. cinerea* can better decompose pectin than other polymers, especially cellulose and hemicellulose. Our results largely corroborate the findings of Finkers et al. (2007) that the mapped QTLs, individually, make a minor contribution to resistance to *B. cinerea*. Thus, this result highlights the importance of the pyramiding of multiple QTLs to achieve strong resistance to *B. cinerea*. However, it should be noted that some of the stem resistance QTLs identified by Finkers et al. (2007) could not be confirmed using the new petiole stub assay, such as the QTLs present in introgression lines 2-1 and 9-2. The discrepancy was not systematically investigated by comparing these genotypes in parallel using both methods, and the mechanisms underlying the discrepancies cannot be explained. Besides stem assays, leaf assays were performed on detached leaves under lab conditions. In the early phase of the project, the inoculum was prepared with PDB medium and the conidial suspension adjusted to a concentration of 300 spores/µL. On LYC4 leaves, almost all the inoculation droplets caused expanding lesions and fully colonized the leaflets after 4 days. Nevertheless, the average size of lesions on LYC4 leaves was significantly smaller than that on MM and the genotypes IL1-3/3-3 and IL4-1 also exhibited smaller lesions with intermediate sizes between LYC4 and MM. We presumed that the nutrient-rich PDB medium was somehow favorable for *B. cinerea*. Host

resistance might be overwhelmed by the aggressiveness of the fungus and as a consequence, the potential difference in disease symptoms between a susceptible and a (partially) resistant genotype would be masked. Derckel et al. (1999) reported a study of the interaction of *B. cinerea* with grape, in which the inoculation with the less aggressive strain T4 enhanced production of defense compounds, whereas the defense responses were significantly compromised or delayed upon inoculation with the more aggressive stain T8. We subsequently used synthetic medium comprising salts, sucrose and phosphate, which allowed us to influence the outcome of fungal infection (Benito et al., 1998). Inoculation of 1000 spores/µL in GB5 medium supplemented with 10 mM sucrose and 10 mM potassium phosphate on LYC4 leaves resulted in high incidence (> 70%) of non-expanding lesions (incompatible interaction), visible as dark black spots under the droplets of inoculum. A similar observation was reported in a different wild tomato species, *S. lycopersicoides*. Leaf inoculation of *B. cinerea* using the same GB5-sucrose-phosphate medium frequently resulted in confined primary lesions which appeared as dispersed black necrosis (Guimarães et al., 2004). By contrast, under the same infection condition, most of the inoculations on MM resulted in expanding lesions defined as compatible interaction (>70%). Remarkably, we observed that *B. cinerea* infection can be promoted by increasing the sucrose concentration. The resistance in LYC4 (high incidence of non-expanding lesions) was compromised by increasing the sucrose concentration. Supplementation of 50 mM sucrose in the inoculum enabled almost all of the inoculation droplets to develop into spreading lesions. On MM, the higher sucrose concentration (50 mM) increased the lesion sizes compared with 10 mM sucrose. How the carbon source concentration in the inoculation droplet facilitates fungal infection after the fungus has penetrated the leaf surface remains unclear. One of the hypotheses might be that a high sugar concentration can affect the expression of genes in *B. cinerea* which play important roles in virulence. A further study focusing on the whole-genome transcriptional analysis in both fungus and host plant upon inoculation with different sucrose concentrations was performed to provide more information on the processes leading to compatible or incompatible interactions between *B. cinerea* and tomato. The results of this transcriptome study are presented in Chapter III.

In this study, spore densities played contrasting roles in *B. cinerea* infection depending on the exact inoculation conditions. As expected, inoculation with low spore densities reduced the aggressiveness of *B. cinerea* infection at high sugar concentration shown as smaller lesion sizes on LYC4. However, counter intuitively, reduction in spore density, such as 30 spores/µL and 100 spores/µL, significantly facilitated the capability to cause expanding lesions compared with 1000 spores/µL when using 10 mM sucrose concentration on LYC4. How the interplay between spore density and sugar concentration affects the percentage of expanding lesions remains to be further studied.

Since *B. cinerea* is a necrotrophic fungus, it benefits from cell death processes in the host plant, and in fact, the induction of host cell death is essential for *B. cinerea* to cause disease (van Kan, 2006). In the incompatible interaction with LYC4, it is unknown how the development of tiny necrotic lesions confines the *B. cinerea* infection. If plant cell death per se is crucial, then what is the difference between a plant cell death process that can stop fungal infection (in the incompatible interaction) and a cell death process which facilitates fungal colonization (in the compatible interaction)? It has been proposed that the outcome of *B. cinerea* interaction depends on a balance between distinct programmed cell death (PCD) pathways, autophagy and apoptosis (Veloso and van Kan, 2018). Although activation of either PCD pathway results in cell death and appearance of necrotic plant tissue, the induction of either pathway has opposing consequences for the host plant (resistance or susceptibility). A mutant from the necrotrophic pathogen *S. sclerotiorum*, a species closely related to *B. cinerea*, triggered autophagic host cell death that resulted in plant resistance (Kabbage et al. 2013). Conversely, *B. elliptica*-induced host cell death displayed features of apoptosis and was shown to be essential for susceptibility (van Baarlen et al., 2007). The manipulation of different outcomes (compatible and incompatible) in LYC4 using different inoculation conditions provides a tool to better understand the interaction between plants and necrotrophs.

According to a previous study, resistance to *B. cinerea* in the tomato ABA-deficient mutant *sitiens* was partially conferred by early and localized defense responses (Asselbergh et al., 2007; Curvers et al., 2010). During the incompatible interaction (non-expanding lesions), accumulation of H_2O_2 and cell wall fortification were observed in the epidermal cells between 4 h and 8 h after *B. cinerea* inoculation (Asselbergh et al., 2007), whereas on wild-type leaves, H_2O_2 accumulation was observed after 24 h in the mesophyll cell layer and it was followed by spreading lesions (Asselbergh et al., 2007). Furthermore, *B. cinerea* infection on *sitiens* can also induce faster and stronger expression of defense-related genes, such as genes encoding pathogenesis-related (PR) proteins. However, in our study, accumulation of H_2O_2 and cell wall fortification represented by phenolic deposition during infection in MM were faster and stronger than LYC4. Besides, inoculation with 10 mM sucrose medium and 50 mM sucrose medium on LYC4 did not exhibit visible difference in DAB and toluidine blue staining at least in the first 24 h.

In conclusion, the main new finding in this work is an experimental protocol by which disease development in the *B. cinerea*-LYC4 interaction can be controlled to either make it compatible or incompatible by simply using different sucrose concentrations in the inoculum. This method provides a convenient tool to study both types of interactions through RNA sequencing. Chapter III will describe comparisons of transcriptional changes, in host plants and *B. cinerea*, during compatible and incompatible interactions.

Materials and Methods

Preparation of inoculum

B. cinerea B05.10 was grown on malt extract agar (MEA) for a week. Conidia were harvested from plates by flooding cultures with 20 mL sterile Milli-Q water and scraping them with a sterile spatula. A suspension containing mycelium and conidia was filtered through glass wool into a 50 mL tube. Subsequently, spore suspension was washed by centrifugation at 1000 revolutions per minute (RPM)/180 relative centrifugal force (RCF) for 10 minutes. Supernatant was discarded and conidia were resuspended in 30-40 mL sterile Milli-Q water. Conidia were counted in a haemocytometer and spore density adjusted to $1x10^7$ spores/mL. To prepare inoculum, GB5 medium including vitamins (Duchefa, The Netherlands) was prepared and supplemented with sucrose at either 10 mM or 50 mM. Potato dextrose broth (PDB) medium was prepared as either 24 g/L (full strength) or 12 g/L (half strength). Inoculum with different spore densities ranging from 30 to 1000 spores/µL were prepared by dilution in the medium from the stock solution (in water).

Stem infection

4-to 5 week-old tomato plants grown in a greenhouse compartment were used for *B. cinerea* inoculation as illustrated in **Figure 1A**. Four compound leaves were detached from plants leaving 1-2 cm petiole stubs. 2 µL of PDB medium containing 2000 spores was pipetted on the wound of the petiole stub. Inoculated plants were then put in the frame covered with plastic sheets for the first two days under high humidity. Disease parameters including lesion size and number of expanding lesions were recorded at 6 and 8 dpi.

Leaf infection

Fully expanded compound leaves were detached and placed in wet florist foam in closed trays under high humidity. Inoculum with different spore densities and media was prepared as above. Two µL droplets were pipetted on the adaxial surface. Four main leaflets from one compound leaf were inoculated with 6 to 8 droplets per leaflet. Disease parameters were recorded from 2 to 4 dpi. Lesion diameters were measured with a digital caliper and lesion areas measured on Image J 1.53e (NIH,USA) from photographs of leaves.

Histochemical staining

In order to investigate the production of reactive oxygen species (ROS), DAB staining was performed as described by Asselbergh et al. (2007) with modifications. Leaf discs were immersed in 5 ml of the freshly prepared DAB solution (1 mg/mL) dissolved in Milli-Q water (pH 3.8 with HCl). To avoid overstaining especially on LYC4, samples were vacuum infiltrated 1-2 times with DAB solution and then were

immediately destained in 40 ml ethanol (96% v/v) heated to 80-90 °C in a water bath for 1-2 h to remove chlorophyll. Afterwards, leaf discs were transferred to a 6-well microplate and washed 2-3 times with Milli-Q water before mounting on a microscope slide. To visualize the fungal hyphae, the leaf discs were counter-stained with trypan blue solution for 1 min. To visualize cell wall fortifications by deposition of phenolics, inoculated leaf discs were stained in 0.05% trypan blue solution in citrate buffer (50 mM, pH 3.5) for 3 to 5 mins. The samples were destained in 40 ml technical ethanol and mounted on microscope slides in 50% glycerol. Bright-field images were taken using a binocular as well as a light microscope.

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Supplementary data

Supplementary Table S1. Results of stem inoculation from three sets of experiments. Mean value of lesion size (longitudinal diameter of the lesion) and growth rate in the ILs and LYC4 were compared with the mean of MM using a student's T-test and significant differences are marked with *P < 0.05 or **P < 0.01.

IL	QTL	Number of inoculations	Number of expanding lesions	Lesion size (mm) at 6 dpi	Lesion size (mm) at 8 dpi	Lesion growth rate (mm/day)
Experiment 1						
1-3/3-3	Rbcq9b	40	38	11.6**	17.3**	4.2**
1-4	Rbcq9b	40	40	19.0**	33.7**	7.9**
2-1	Rbcq2	40	40	38.4*	63.8	13.0
4-1	Rbcq4a	40	40	20.8*	34.9**	7.6**
9-1	Rbcq9a	40	40	21.7**	37.5**	7.9**
11-2	Rbcq11	40	30	11.1**	16.4**	3.2**
12-3	Rbcq9b & Rbcq12	40	40	26.6	44.9*	9.1**
LYC-4		40	4	7.0**	10.5**	2.3**
MM		40	40	29.3	55.0	12.8
Experiment 2						
1-3/3-3	Rbcq9b	40	19	8.5**	15.3**	3.8**
1-4	Rbcq9b	40	22	9.8**	21.0**	6.4**
2-1	Rbcq2	40	30	24.4	45.6	11.0
4-1	Rbcq4a	28	13	11.9**	24.0**	6.6**
9-2	Rbcq9b	40	10	14.7**	38.3*	12.0
11-2	Rbcq11	40	17	8.0**	13.1**	4.1**
12-3	Rbcq9b & Rbcq12	36	25	21.9	35.8**	7.5**
LYC-4		52	1	4.2**	4.5**	-
MM		60	54	25.5	50.4	12.5
Experiment 3						
1-3/3-3	Rbcq9b	40	5	5.7**	6.6**	-
1-4	Rbcq9b	40	9	19.9*	31.1*	6.3
2-1	Rbcq2	40	30	34.0	51.1	8.8
4-1	Rbcq4a	28	22	22.2	35.6*	7.7
9-1	Rbcq9a	24	20	27.1	42.3	8.0
9-2	Rbcq9b	36	13	32.13	48.4	8.1
11-2	Rbcq11	40	17	8.4**	10.7**	3.1**
12-3	Rbcq9b & Rbcq12	20	6	18.4*	26.3**	3.9**
LYC-4		52	1	4.8*	6.0**	-
MM		40	14	32.7	52.8	10.1

Supplementary Table S2. Results of leaf inoculation from three sets of experiments. Mean value of lesion size (diameter of lesion) and growth rate in the ILs and LYC4 were compared with the mean of MM using a student's T-test and significant differences are marked with *P < 0.05 or **P < 0.01.

IL	QTL	Lesion size (mm) at 48 hpi	Lesion size (mm) at 72 hpi	Lesion growth rate (mm/day)
Experiment 1				
1-3/3-3	Rbcq9b	6.89 **	12.93	6.04
1-4	Rbcq9b	6.59	13.45	6.86
2-1	Rbcq2	7.49 **	14.41 **	6.93
4-1	Rbcq4a	6.39	12.58	6.18
9-1	Rbcq9a	7.28 **	14.50 **	7.22
11-2	Rbcq11	8.69 **	14.72 **	6.04
12-3	Rbcq9b & Rbcq12	6.56	12.51	5.96
LYC-4		3.99 **	8.78 **	4.78
MM		6.02	12.70	6.68
Experiment 2				
1-3/3-3	Rbcq9b	5.9 **	11.5 **	5.6 **
1-4	Rbcq9b	7.2	14.5 **	7.3 **
2-1	Rbcq2	8.0 **	15.9 **	7.9 **
4-1	Rbcq4a	5.7 **	11.1 **	5.4 **
9-2	Rbcq9b	8.0 **	14.5	6.5
11-2	Rbcq11	7.9 **	14.6 **	6.7
12-3	Rbcq9b & Rbcq12	6.4 **	12.1 **	5.7 **
MM		7.3	13.7	6.4
Experiment 3				
1-3/3-3	Rbcq9b	7.3**	10.8**	3.9**
1-4	Rbcq9b	9.2	13.9	4.7
2-1	Rbcq2	10.2**	15.8**	5.5**
4-1	Rbcq4a	7.9**	11.2**	4.3
9-1	Rbcq9a	9.9*	15.4**	5.6**
9-2	Rbcq9b	9.4	14.4	5.1
11-2	Rbcq11	9.6	14.3	4.8
12-3	Rbcq9b & Rbcq12	9.7	14.6	5.3*
LYC-4		7.6**	11.2**	5.3
MM		9.4	14.2	4.7

Chapter 3

Transcriptome analysis of compatible and incompatible interactions between *Botrytis cinerea* and tomato

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Abstract

The necrotrophic fungal pathogen *Botrytis cinerea* can cause grey mould disease on many plants including cultivated tomato *Solanum lycopersicum*. Quantitative resistance to *B. cinerea* has been identified in the tomato wild relative *S. habrochaites* accession LYC4. Infection on LYC4 using inoculum containing 1000 spores/µL in Gamborg's B5 (GB5)-10 mM sucrose-10 mM phosphate medium can lead to high incidence of incompatible interaction, whereas compatible interaction was observed for infection on MM under the same infection condition or on LYC4 with increased sucrose concentration (50 mM, **Chapter II**). In this chapter an RNA sequencing (RNA-seq) approach was adopted to study the transcriptional reprogramming, both in the fungus and the plant, during the compatible and incompatible interactions between tomato and *B. cinerea*. Fungal gene expression remained similar during the compatible and incompatible interactions on LYC4 until 16 hpi, while at 24 hpi we observed in the incompatible interaction the induction of a large number of genes that were putatively under catabolite repression. The relief of catabolite repression at 24 hpi was substantiated in a fungal sample grown in 10 mM sucrose *in vitro*. *B. cinerea* displayed very different transcription patterns during infection on LYC4 as compared with MM infection, with the largest number of differentially expressed genes (2081) observed at 24 hpi. Moreover, *B. cinerea* genes such as *BcatrB* and *Bclcc2* involved in detoxification of plant antimicrobial compounds displayed substantially higher expression levels on LYC4 than on MM especially in the early infection stages (12 hpi). By contrast, α-tomatine-responsive genes in *B. cinerea*, for instance, *BcTom1* encoding tomatinase, exhibited delayed up-regulation on LYC4 in comparison with MM infection indicating delayed induction of plant cell death in LYC4. The delay in infection in LYC4 was also reflected by the later and weaker plant transcription response in LYC4 than in MM. Finally, we observed that LYC4 and MM differed in the expression of a large number of genes (>8000) even in the absence of *B. cinerea*.

Introduction

Botrytis cinerea is one of the most well-studied necrotrophic fungal pathogens that causes grey mould disease on more than 1400 plant species (Elad et al., 2016). As a necrotrophic pathogen, *B. cinerea* can actively kill the plant cells through secretion of cell death inducing molecules including secondary metabolites (botrydial and botcinic acid) and phytotoxic proteins (Choquer et al., 2007; van Kan, 2006). The genome of *B.* cinerea contains a large number of genes encoding carbohydrate-active enzymes and auxiliary proteins (CAZymes) of which at least 132 are potential plant cell wall degradation enzymes (CWDEs) that contribute to decomposition and consumption of plant biomass (Amselem et al., 2011; Valero et al, 2019). In addition, several *B. cinerea* CWDEs including xylanase BcXYL1 (Yang et al., 2018) BcXyn11A (Noda et al., 2010), glucosidase BcGS1 (Zhang et al., 2015), xyloglucanase BcXYG1 (Zhu et al., 2017) and endopolygalacturonases (Kars et al., 2005; Poinssot et al., 2003; Zhang et al., 2014) were reported to be able to induce host cell death and defense responses. Some of these CAZymes play an important role in *B. cinerea* infection. For instance, reduced virulence has been observed in *B. cinerea* mutants lacking BcPG2 (Kars et al., 2005), BcPG1 (ten Have et al., 1998), BcXyn11A (Noda et al., 2010) and BcXYL1 (Yang et al., 2018), separately. The expression of some CAZyme genes in filamentous fungi can be repressed in the presence of preferable carbon sources such as glucose and sucrose, which is referred to as carbon catabolite repression (CCR) (Adnan et al., 2018; Tudzynski et al., 2000). The mechanism of CCR has been extensively studied in several model fungi, and relies on the binding of a conserved negative transcriptional regulator CreA to the promoter of repressed genes (Adnan et al., 2018; Cubero and Scazzocchio, 1994; Suto and Tomita, 2001). In *B. cinerea*, multiple CreA binding sites were present in the promoter sequences of several members of the endopolygalacturonase (*Bcpg*) gene family and the *Bcpg4* gene was indeed found to be under CCR when growing in medium containing a high concentration of glucose (Wubben et al., 1999). The expression of the *B. cinerea* xylanase encoding gene *Bcxyn10A* was also regulated by CCR (García et al., 2017).

Plant pathogens can encounter an array of antimicrobial secondary metabolites produced by the host during plant infection. To mitigate the effect of the toxic compounds, plant pathogens including *B. cinerea* employ different active strategies to deal with antifungal compounds (Westrick et al., 2021). Membrane efflux pumps including ABC transporters and major facilitator superfamily proteins can mediate the efflux of toxic compounds from cytoplasm to the extracellular space (Perlin et al., 2014). Secretion of enzymes such as laccase and tomatinase to directly modify the antifungal substances aiming to reduce their toxicity was also studied in *B. cinerea* (Buddhika et al., 2021; Schouten et al., 2008; Chapter V). In general,

mechanisms of tolerance to antifungal substances are activated at the transcriptional level by these substances, even when they have a different mode of action. Tolerance to antifungal substances produced by host plants plays an important role in the virulence of *B. cinerea*. For instance, the efflux of camalexin and glucosinolate-breakdown products by the BcatrB and BcmfsG transporters, respectively, is required for the full virulence of *B. cinerea* in Arabidopsis (Stefanato et al., 2009; Vela-Corcía et al., 2019). Hydrolytic deglycosylation of the tomato saponin α-tomatine by BcTom1 is also required for virulence of *B. cinerea* on tomato (**Chapter V**).

Tomato is a member of the *Solanaceae* family and is originally native to middle and south America. The cultivated tomato (*Solanum lycopersicum*) was introduced into Europe after the Spanish conquest of Mexico in 1521. It is one of the most cultivated vegetable crops worldwide providing an important source of nutrients. Apart from its economic importance, tomato is also a model plant for genetic research especially regarding plant-microbe interactions (Arie et al., 2006; Abbasi et al., 2021). The pre- and post-harvest cultivation of tomato is affected by grey mould disease because of its high susceptibility to *B. cinerea* (Williamson et al., 2007). Considerable efforts have been made in the past two decades searching for genetic resources with better performance against *B. cinerea* to improve the tomato breeding. However, only quantitative resistance has been uncovered in several wild *Solanum* species including *S. habrochaites* (ten Have et al., 2006), *S. neorickii* (Finkers et al., 2008), and *S. lycopersicoides* (Guimarães et al., 2004). The genetic mapping in an introgression population derived from *S. habrochaites* accession LYC4 revealed 10 QTLs, all making minor contributions to the partial resistance to *B. cinerea* in LYC4 (Finkers et al., 2007). So far major resistance QTLs have not been identified in the wild relatives of tomato and this current situation necessitates the pyramiding of multiple resistance QTLs during tomato breeding to substantially increase the resistance to grey mould disease.

The molecular basis of partial resistance to *B. cinerea* in the wild *Solanum* species has not been unraveled. This is partially because of the low resistance contribution of single QTLs reported and because of the divergence of genome structures between cultivated tomato and its wild relatives, which hampered genetic approaches such as fine mapping. Transcriptome analysis using RNA-sequencing (RNA-seq) has been demonstrated to be a useful approach to investigate the transcriptional reprogramming during plant-microbe interactions, in both host plants and pathogenic microbes, at the whole genome level (AbuQamar et al., 2016). Therefore, in this study, we analyzed the transcriptional profiles of tomato and *B. cinerea* during compatible and incompatible interactions, resulting in host susceptibility and resistance, respectively. The aim of this study is to identify key factors in both plant and fungus that play a role in the

tomato-*B. cinerea* interaction to increase our understanding of the molecular mechanisms underlying host resistance or susceptibility, as well as fungal virulence.

Results

Inoculation and sampling

Compatible and incompatible *Solanum-B. cinerea* interactions were experimentally achieved as described in **Chapter II**. In brief, leaves of *S. lycopersicum* cultivar Moneymaker (MM) were susceptible to *B. cinerea* isolate B05.10 (i.e. compatible interaction) when inoculated in GB5 medium regardless of the sucrose concentrations tested (10 or 50 mM). By contrast, leaves of the wild tomato relative *S. habrochaites* accession LYC4 were susceptible to B05.10 when 50 mM sucrose was used in the inoculum (compatible interaction), but were resistant when 10 mM sucrose was used in otherwise identical conditions (incompatible interaction). Inoculations were performed to obtain samples from two compatible interactions (MM with 10 mM sucrose and LYC4 with 50 mM sucrose) and one incompatible interaction (LYC4 with 10 mM sucrose).

mRNA samples were generated from *B. cinerea*-infected leaves of all three combinations at 12, 16 and 24 hours post inoculation (hpi). Mock-inoculated tomato leaves, as well as *B. cinerea* liquid cultures (grown in the inoculation medium with either 10 or 50 mM sucrose), sampled at the same three time points were included as controls. All samples were used for generating strand-specific libraries and sequenced at read depths varying from 5 Million to 100 Million reads per sample. Sequence reads were mapped, separately, to the *B. cinerea* and the tomato genome and read counts for the gene models were performed to determine transcript levels. The coding of samples and datasets that is used throughout this chapter is provided in **Table 1**.

Table 1. Abbreviations and treatment conditions for *B. cinerea* and plant samples.

Sample abbreviations	Time points (h or hpi)	Treatment description
Bc10S0A, B Bc10S12A, B Bc10S16A,B Bc10S24A, B	0, 12, 16, 24 (h)	Fungal gene expression of samples from *B. cinerea* liquid culture grown in GB5-10 mM sucrose-10 mM phosphate medium
Bc50S0A, B Bc50S12A, B Bc50S16A, B Bc50S24A, B	0, 12, 16, 24 (h)	Fungal gene expression of samples from *B. cinerea* liquid culture grown in GB5-50 mM sucrose-10 mM phosphate medium
BcLI1012A, B,C BcLI1016A, B ,C BcLI1024A, B, C	12, 16, 24 (hpi)	Fungal gene expression of samples from LYC4 leaves inoculated with *B. cinerea* spores in GB5-10 mM sucrose-10 mM phosphate inoculum
BcLI5012A, B, C BcLI5016A, B, C BcLI5024A, B, C	12, 16, 24 (hpi)	Fungal gene expression of samples from LYC4 leaves inoculated with *B. cinerea* spores in GB5-50 mM sucrose-10 mM phosphate inoculum
BcMI1012A, B, C BcMI1016A, B, C BcMI1024A, B, C	12, 16, 24 (hpi)	Fungal gene expression of samples from MM leaves inoculated with *B. cinerea* spores in GB5-10 mM sucrose-10 mM phosphate inoculum
LI1012A, B, C LI1016A, B, C LI1024A, B, C	12, 16, 24 (hpi)	Plant gene expression in LYC4 leaf samples inoculated with *B. cinerea* spores in GB5-10 mM sucrose-10 mM phosphate inoculum
LI5012A, B, C LI5016A, B, C LI5024A, B, C	12, 16, 24 (hpi)	Plant gene expression in LYC4 leaf samples inoculated with *B. cinerea* spores in GB5-50 mM sucrose-10 mM phosphate inoculum
MI1012A, B,C MI1016A, B, C MI1024A, B, C	12, 16, 24 (hpi)	Plant gene expression in MM leaf samples inoculated with *B. cinerea* spores in GB5-10 mM sucrose-10 mM phosphate inoculum
LM0A, B, C LM12A, B, C LM16A, B, C LM24A, B, C	0, 12, 16, 24 (hpi)	Plant gene expression in LYC4 leaf samples under mock treatment
MM0A, B, C MM12A, B, C MM16A, B, C MM24A, B, C	0, 12, 16, 24 (hpi)	Plant gene expression in MM leaf samples under mock treatment

Transcriptional reprogramming in *Botrytis cinerea* during compatible and incompatible interactions

The count files of *B. cinerea* samples contained 11699 Ensembl IDs. Transcript isoforms and genes that have less than 10 read counts in every sample were regarded as non-informative and were removed from further analysis. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) using normalized reads was performed to characterize the connection of transcriptional profiles in *B. cinerea* with different biological conditions including sucrose concentration in the inoculum, time course during infection, and inoculation on different host species (**Figure 1**). PCA revealed the variation of fungal transcript profiles between the *in vitro* culture and plant infection (**Figure 1A**). Besides, fungal gene expression in MM and LYC4 inoculated in 10 mM sucrose exhibited large separation only at 24 hpi. The Bc10S24A sample clearly deviated from other *in vitro* samples, as can be observed in the PCA plot.

The dendrogram produced by HCA contained 4 main clusters, with the *in vitro* cultured samples (clusters A and cluster D) clearly separating from the infection samples (clusters B and C). Cluster A comprises samples from ungerminated *B. cinerea* spores, which showed a very different transcriptional profile from the other samples in which the fungus had germinated. The fungal transcript profiles at 24 hpi on LYC4 and MM separated from the early time points (12 hpi, 16 hpi), except for BcLI5024 samples which were collected in LYC4 at 24 hpi in 50 mM sucrose. During *in vitro* growth, one replicate of the 10 mM sucrose sample Bc10S24A (in cluster D) stood out from other samples including its biological replicate Bc10S24B.

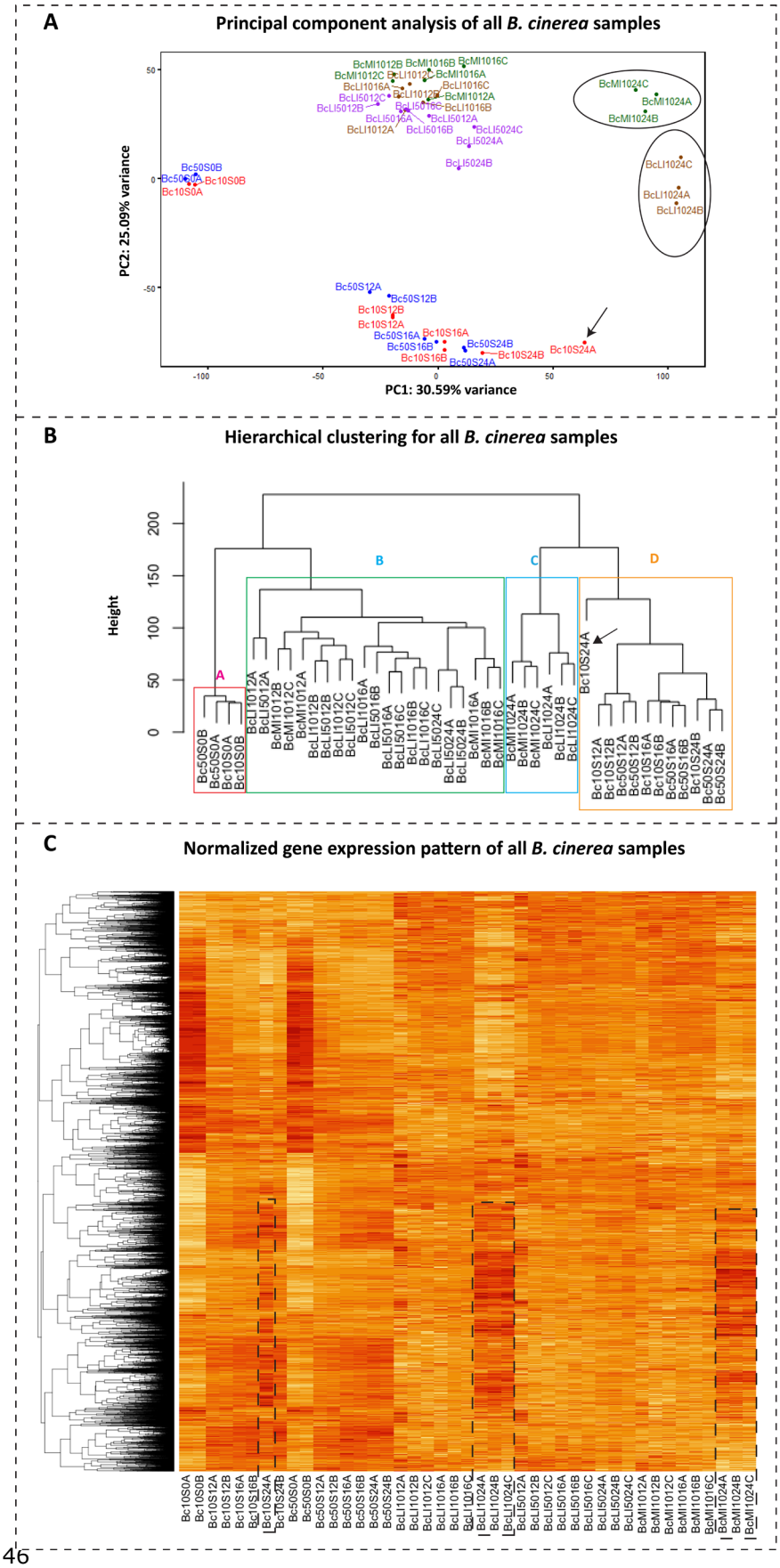


Figure 1. Overview of *B. cinerea* gene expression profiles. Principal component analysis for all *B. cinerea* samples (A); Hierarchical clustering dendrogram for all *B. cinerea* samples (B). The four rectangular boxes represent the clusters of samples if the dendrogram is cut at the height of 150; Heatmap for normalized gene expression patterns of all *B. cinerea* samples (C). The dendrogram on the left margin reflects the hierarchical clustering result of all *B. cinerea* genes that have minimum normalized count values larger than 30 across samples. The 43 columns of the heatmap record all *B. cinerea* gene expression levels within a sample. Each row of the heatmap is made up of multiple cubes denoting the expression level of a single gene.

The global view of normalized gene expression patterns among all *B. cinerea* samples was displayed in a heatmap (**Figure 1C**). Samples collected at 24 hpi including Bc10S24A, BcMI1024A, B, C and BcLI1024A, B, C exhibited up-regulation of a similar array of genes (**Figure 1C**). Overall, fungal gene expression either during infection or *in vitro* growth was not strongly affected by sucrose concentrations in the early stage (12 hpi and 16 hpi). Samples collected at 24 hpi exhibited strong variance as compared with the samples collected at earlier time points indicating the occurrence of drastic transcriptional reprogramming. One biological replicate of *in vitro* grown sample at 24 h (Bc10S24A) separated from all other *in vitro* grown samples which suggested that in this sample, the fungus experienced a different physiological status.

***Botrytis cinerea* displayed different transcript profiles between infection on MM and LYC4**

The differentially expressed genes (DEGs) between biological conditions were analyzed by DESeq2 for 28 pairwise sample comparisons, of which a subset is displayed in **Figure 2**.

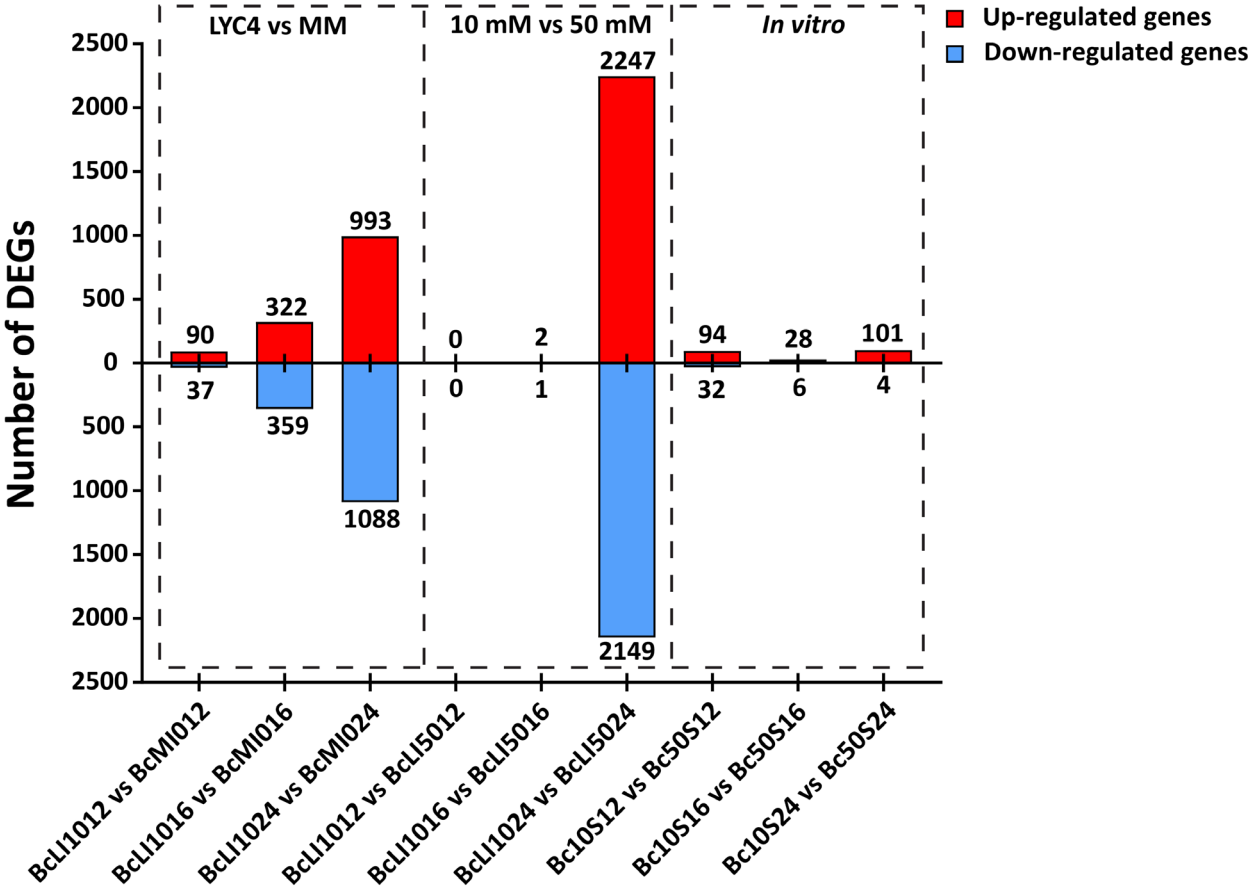


Figure 2. Number of up-and down-regulated *B. cinerea* genes during plant infection and *in vitro* growth.

B. cinerea exhibited different expression patterns after inoculation of LYC4 and MM in 10 mM sucrose and the number of DEGs significantly increased over time during the infection. For instance, there were only 90 up-regulated genes and 37 down-regulated genes between infection on LYC4 and infection on MM at 12 hpi, whereas numbers of DEGs at 24 hpi increased to 993 up-regulated and 1088 down-regulated genes, respectively.

To further characterize the fungal transcriptional variation between infection of different host species, Gene Ontology (GO) term enrichment analysis was carried out on the DEGs. Strikingly, no functional enrichment was identified in the up-regulated genes (993) in LYC4 infection as compared with MM infection. Among the down-regulated genes during LYC4 infection, a large number of GO terms were enriched at 16 hpi and 24 hpi that are related to different processes, such as macromolecule biosynthetic process (GO:0034645) and organonitrogen compound biosynthetic process (GO:1901566) and translation (GO:0006412) (**Figure 3**). The enrichment analysis of Molecular Function (MF) terms revealed that the down-regulated genes were enriched for genes associated with RNA binding (GO:0003723) and translation regulator activity (GO:0045182) during 10 mM sucrose infection on LYC4 (incompatible interaction) as compared with a compatible interaction on MM (**Figure 3**).

Genes for carbohydrate consumption were uniquely up-regulated during 10 mM sucrose infection at 24 hpi

There were no significant changes in *B. cinerea* gene expression on LYC4 upon inoculation in 10 mM and 50 mM sucrose at 12 hpi and only three DEGs were detected at 16 hpi. However, enormous variation in transcript profiles was observed at 24 hpi with 4396 DEGs, almost equally divided over up- and downregulated genes (**Figure 2**), accounting for 37.5% of the annotated genes in the *B. cinerea* genome. In GO enrichment analysis of the DEGs, the Biological Process (BP) term carbohydrate metabolic processes (GO:0005975) and MF terms including hydrolase activity (GO:0004553), carbohydrate binding (GO:0030246) and cellulose binding (GO:0030248) were enriched in the up-regulated genes in BcLI1024 as compared with BcLI5024 (**Figure 4**).

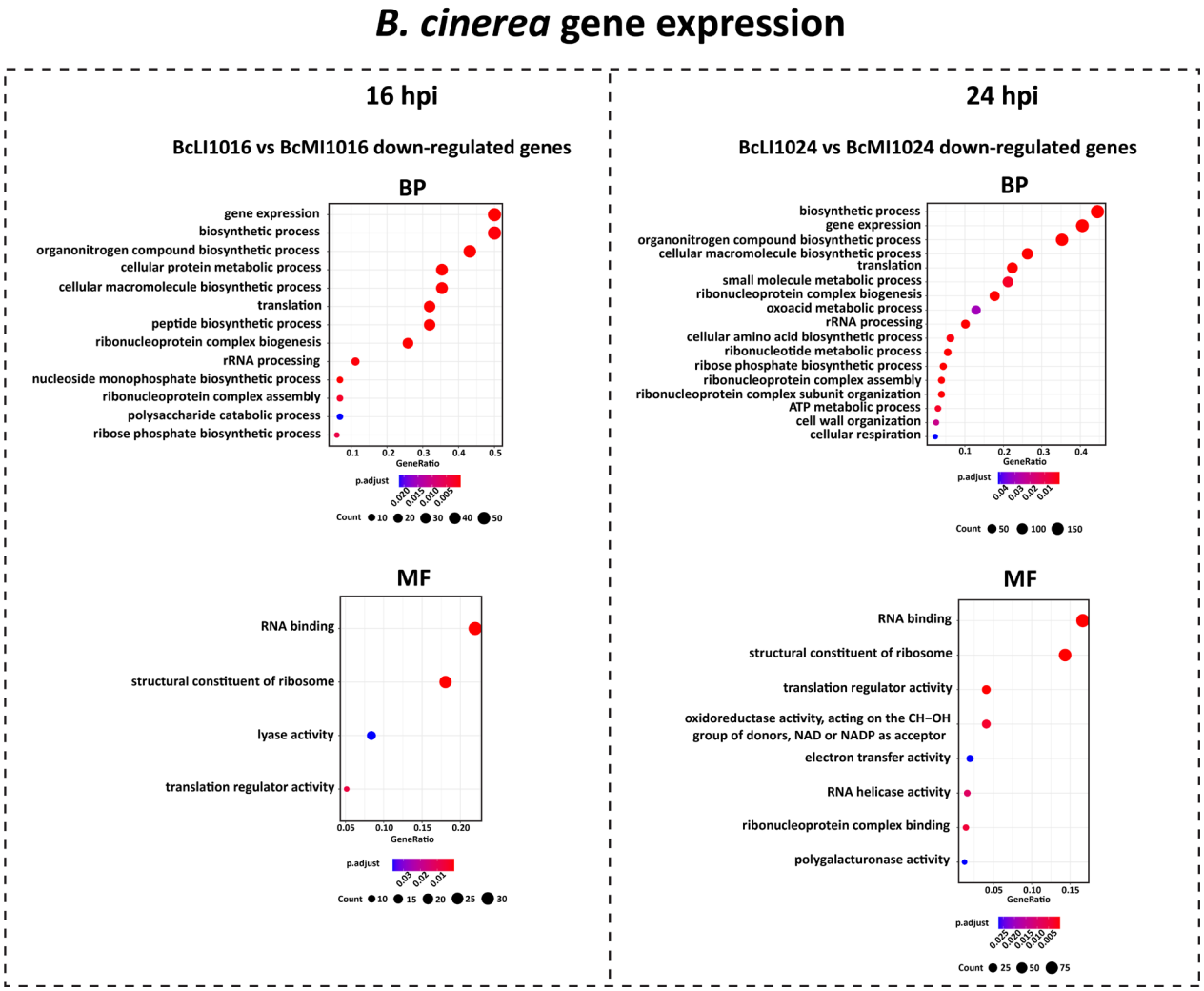


Figure 3. Functional GO enrichment analysis of *B. cinerea* DEGs between infection on LYC4 and MM at different timepoints. Only genes that were down-regulated during LYC4 infection as compared with MM infection, displayed significant enrichment of GO terms at 16 hpi and 24 hpi.

We then compared 10 mM sucrose infection on LYC4 or MM with 50 mM sucrose infection on LYC4 at 24 hpi to determine the common effect of sugar concentration on fungal gene expression during infection of different hosts. Although infecting distinct tomato genotypes (MM and LYC4), 1250 genes and 828 *B. cinerea* genes were commonly up- or down-regulated, respectively, in 10 mM sucrose infection (BcLI1024 and BcMI1024) as compared with 50 mM sucrose infection on LYC4 (BcLI5024) (**Figure 5**). In the overlapping sets of up-regulated genes, GO terms associated with utilization of carbohydrates were overrepresented (**Figure 5**).

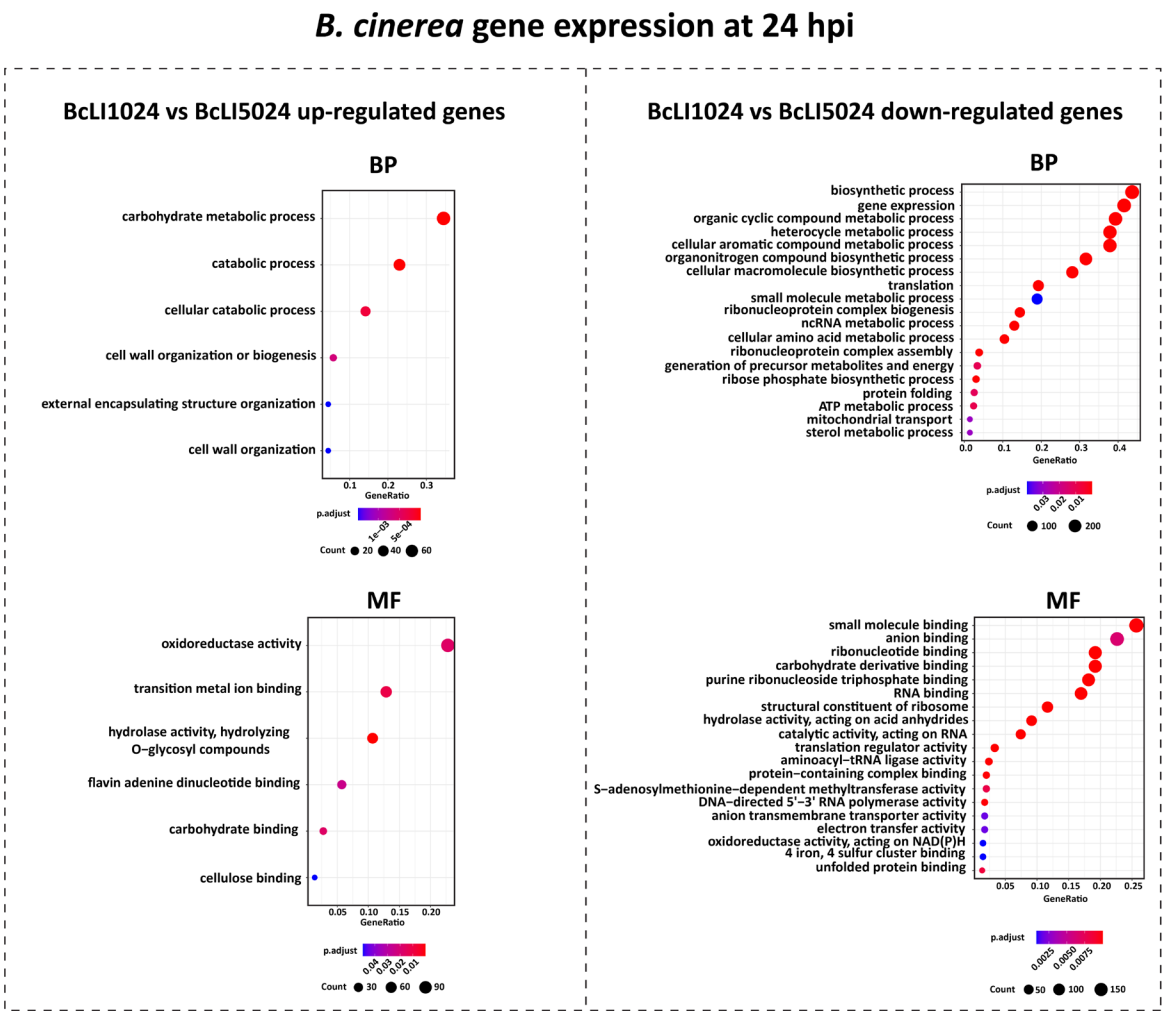


Figure 4. Functional enrichment of *B. cinerea* DEGs between 10 mM and 50 mM sucrose infection on LYC4 at 24 hpi.

Based on these observations, we decided to analyze expression profiles of carbohydrate utilization genes in more detail, across infection time points and from *in vitro* cultures. A large number of genes encoding glycosyl hydrolases and sugar transporters were lowly expressed at early time points (12 hpi and 16 hpi) both during tomato infection (MM and LYC4) as well as *in vitro* growth in liquid medium. For many of these glycosyl hydrolase and sugar transporter genes, an abrupt transition to high expression was observed exclusively for 10 mM sucrose infection on both LYC4 and MM at 24 hpi, but not in any 50 mM sucrose samples. Specifically, there was a strong up-regulation in BcLI1024 and BcMI1024 (as compared to BcLI5024) of all the genes participating in consumption of the pectin monomer D-galacturonic acid: the membrane transporters *Bchxt15* (Bcin07g03780) and *Bchxt19* (Bcin01g07270), the catabolic pathway genes *Bclga1* (Bcin03g01490), *Bclgd1* (Bcin01g09450), *Bcgar1* (Bcin06g04660), *Bcgar2* (Bcin03g01500) and *Bcglr1* (Bcin03g00240), and the transcriptional regulator *BcgaaR* (Bcin09g00170) (**Figure 6A**).

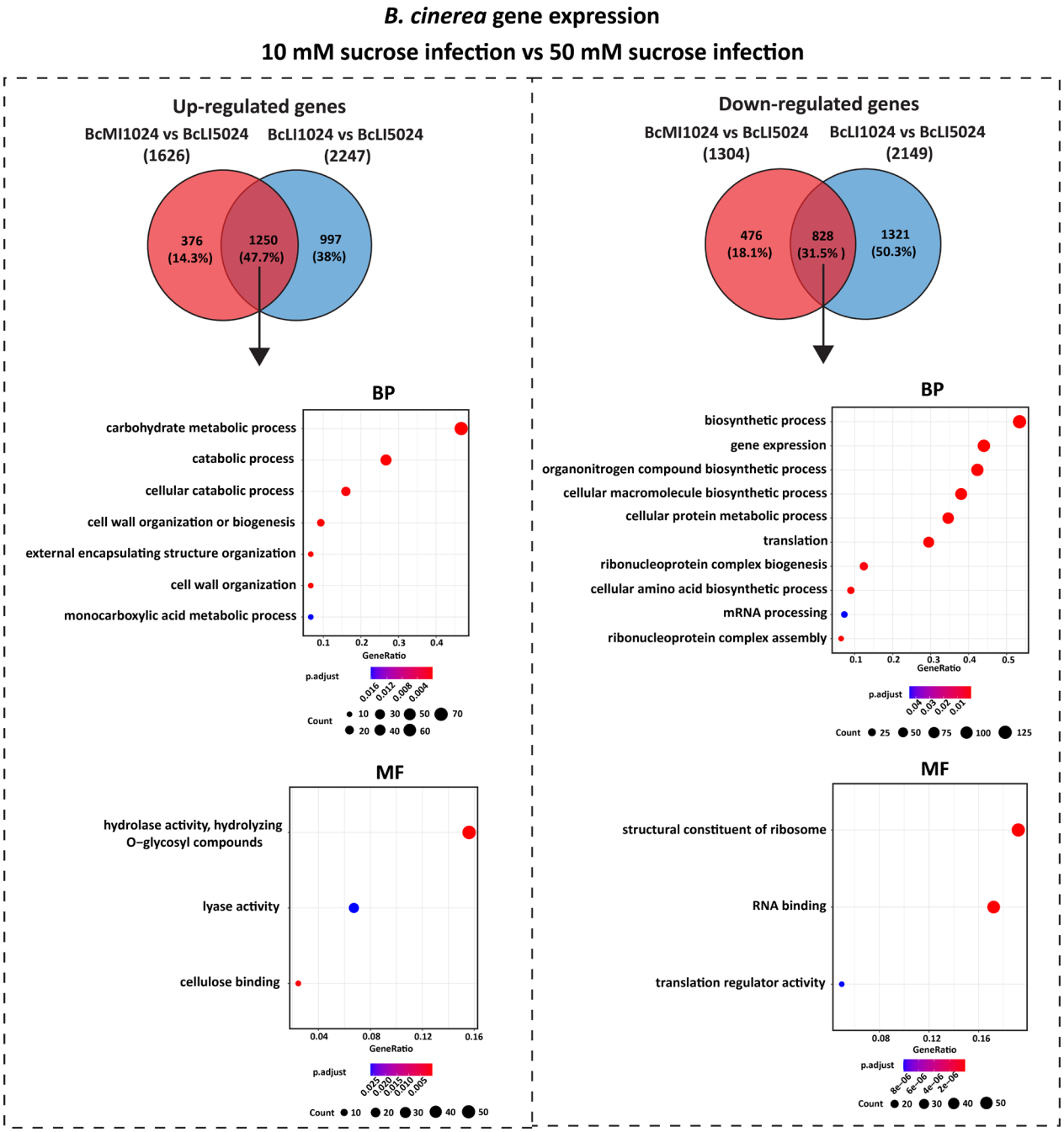


Figure 5. Common up- and down-regulated *B. cinerea* genes between 10 mM sucrose infection and 50 mM sucrose infection on LYC4 and MM.

Proteins containing CFEM domains (Common in Fungal Extracellular Membrane proteins) are specific to fungi (Kulkarni et al., 2003) and also displayed interesting expression patterns. In *B. cinerea* two genes encoding CFEM proteins have been characterized. BcCFEM1 (Bcin10g02180) contains a glycosyl-phosphatidylinositol (GPI) anchoring motif, it can induce plant chlorosis and was reported to be required for virulence (Zhu et al., 2017). Bcin07g03260 encodes a membrane CFEM protein, deletion of which also

resulted in reduced virulence on tomato (Arya et al., 2020). However, the genome of *B. cinerea* strain B05.10 encodes 13 proteins containing a CFEM domain, seven of which displayed considerable expression during infection (**Figure 6B**). Strikingly, *Bccfem1* (Bcin10g02180) and gene Bcin08g00940 were barely expressed at early stages (12 hpi and 16 hpi), but their transcript levels drastically increased at 24 hpi both on LYC4 and MM, but only when inoculation was performed in 10 mM sucrose. Three *CFEM* genes (Bcin01g05020, Bcin04g06730, Bcin09g02270), displayed very high expression levels during plant infection and peaked at different timepoints (**Figure 6B**).

A **D-galacturonic acid catabolic pathway**

Accession number	Gene ID	Pfam annotation	Fold change (log2) P-value <0.05 BcLI10/BcMI10 12 HPI	16 HPI	24 HPI	BcLI10/BcLI50 12 HPI	16 HPI	24 HPI	Normalized Counts BcMI10 12 HPI	16 HPI	24 HPI	BcLI10 12 HPI	16 HPI	24 HPI	BcLI50 12 HPI	16 HPI	24 HPI
Bcin07g03780	Bchxt15	Sugar (and other) transporter	n.s.	n.s.	n.s.	n.s.	n.s.	3.5	4	51	1139	7	15	236	5	11	21
Bcin01g07270	Bchxt19	Sugar (and other) transporter	n.s.	n.s.	n.s.	n.s.	n.s.	9.5	3	69	6543	13	7	3754	15	29	5
Bcin03g01490	Bclga1	Dihydrodipicolinate synthetase family	n.s.	n.s.	n.s.	n.s.	n.s.	4.7	44	274	8050	43	100	3245	42	81	123
Bcin01g09450	Bclgd1	Enolase C-terminal domain-like	n.s.	n.s.	n.s.	n.s.	n.s.	3.0	294	475	4256	248	381	2856	231	316	357
Bcin06g04660	Bcgar1	Aldo/keto reductase family	n.s.	n.s.	n.s.	n.s.	n.s.	1.5	556	671	2055	560	878	1483	652	675	530
Bcin03g01500	Bcgar2	Oxidoreductase family	n.s.	n.s.	n.s.	n.s.	n.s.	4.3	36	145	3390	53	65	1334	29	52	67
Bcin03g00240	Bcglr1	Aldo/keto reductase family	n.s.	n.s.	n.s.	n.s.	n.s.	6.1	11	21	704	9	17	2894	17	33	41
Bcin09g00170	BcgaaR	Fungal Zn(2)-Cys(6) binuclear cluster domain	n.s.	n.s.	n.s.	n.s.	n.s.	3.2	79	329	1096	97	201	1546	77	121	165

Normalized Counts: 2000 4000 6000 8000

B **CFEM**

Accession number	Gene ID	Pfam annotation	Fold Change(log2) P-value <0.05 BcLI10/BcMI10 12 HPI	16 HPI	24 HPI	BcLI10/BcLI50 12 HPI	16 HPI	24 HPI	Normalized Counts BcMI10 12 HPI	16 HPI	24 HPI	BcLI10 12 HPI	16 HPI	24 HPI	BcLI50 12 HPI	16 HPI	24 HPI
Bcin10g02180	Bccfem1	CFEM domain	n.s.	n.s.	1.0	n.s.	n.s.	8.2	2	36	3019	4	32	6000	14	10	20
Bcin15g04520	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	3.4	1	5	8	2	2	11	1	3	
Bcin15g02580	-	CFEM domain	n.s.	n.s.	1.9	n.s.	n.s.	0.7	350	346	160	326	471	604	302	442	364
Bcin14g05390	-	CFEM domain	n.s.	n.s.	1.5	n.s.	n.s.	2.8	251	272	447	173	256	1276	153	192	178
Bcin13g03160	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	-3.0	4	9	10	1	3	4	0	4	30
Bcin09g02270	-	CFEM domain	n.s.	0.7	1.1	n.s.	n.s.	1.5	1813	1245	2189	1831	1997	4666	1689	1907	1659
Bcin08g00940	-	CFEM domain	n.s.	n.s.	1.6	n.s.	n.s.	11.2	0	1	793	0	0	2325	3	0	1
Bcin07g03260	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	10	18	20	13	11	39	13	11	41
Bcin06g01950	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	1	1	0	1	1	0	0	0
Bcin05g02420	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	4	9	20	0	2	9	1	7	5
Bcin04g06730	-	CFEM domain	n.s.	n.s.	1.2	n.s.	n.s.	n.s.	2855	2041	711	2406	3448	1674	3260	5169	2152
Bcin01g05020	-	CFEM domain	n.s.	0.8	n.s.	n.s.	n.s.	1.4	2358	6623	1534	933	5527	2406	812	5186	894
Bcin01g01980	-	CFEM domain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	14	26	27	8	26	14	6	23	14

Normalized Counts: 0 2000 4000 6000

Figure 6. Expression profile of D-Galacturonic Acid catabolic pathway genes (A) and CFEM domain protein-encoding genes in *B. cinerea* (B) during LYC4 and MM infection using different sucrose concentrations.

Compatible interactions of *B. cinerea* on LYC4 and MM exhibited shared common transcriptional features distinct from the incompatible interaction on LYC4

We then analyzed the similarity in the fungal transcription patterns during compatible interactions either on MM, or on LYC4 (conferred by 50 mM sucrose infection). As shown in **Figure 7**, BcLI5024 and BcMI1024 (both compatible interactions) shared 798 genes with higher expression levels than in BcLI1024 (incompatible interaction). From these genes GO terms associated with biosynthetic process, gene

expression, translation and ATP metabolic process were enriched. Furthermore, a large number of house-keeping genes encoding mRNA recognition motif-containing proteins, ribosomal proteins, mitochondrial proteins and ATP synthases were collectively down-regulated in BcLI1024, suggesting a cessation of primary metabolic processes in the incompatible interaction.

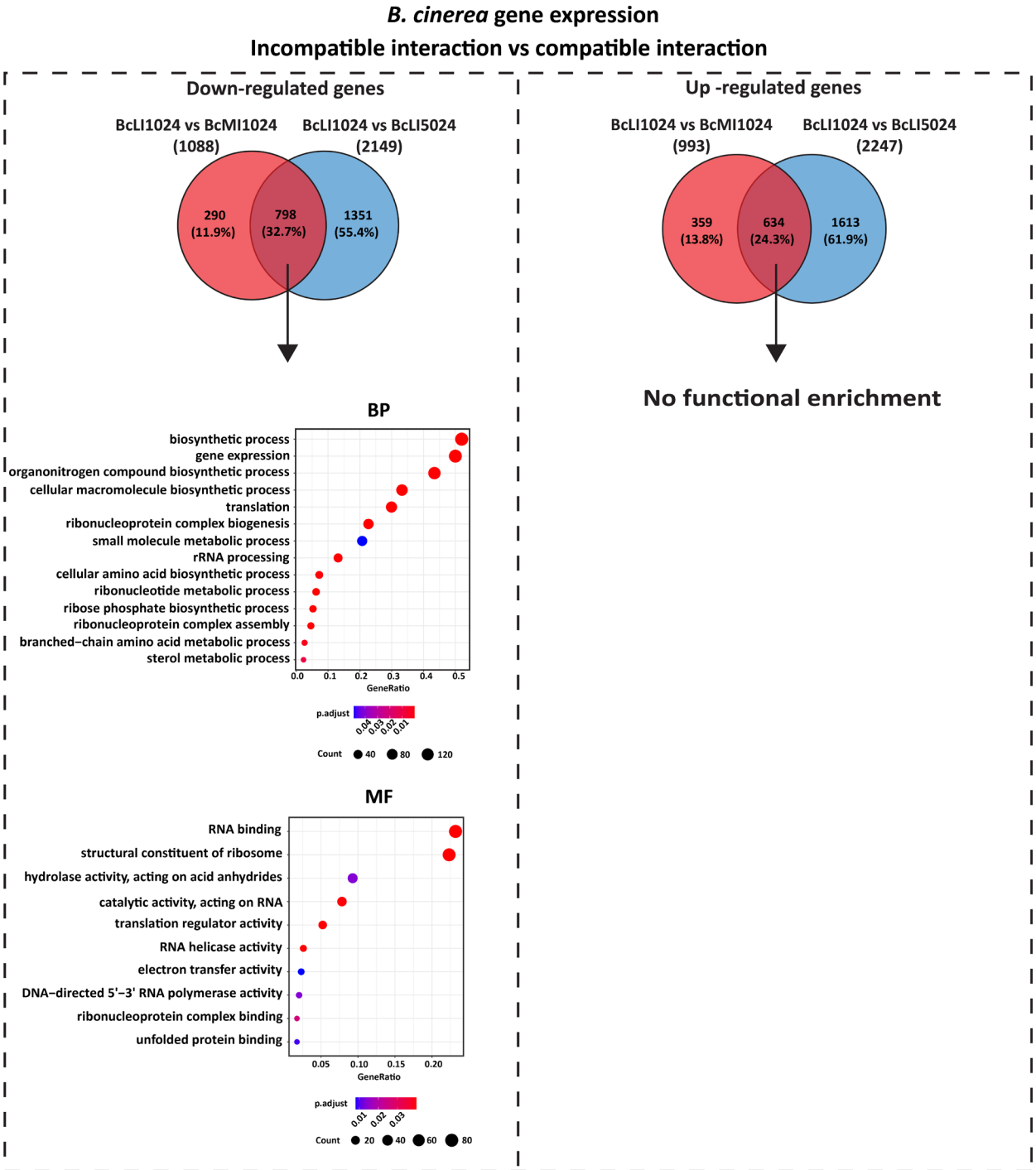
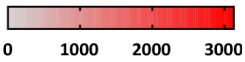


Figure 7. Common up- and down-regulated *B. cinerea* genes between compatible and incompatible interactions on LYC4 and MM.

There were 634 genes which exhibited higher expression uniquely in the incompatible interaction BcLI1024 (as compared to both compatible interactions) but no enrichment of GO terms was identified among those genes (**Figure 7**). Within this set of 634 genes, *Bcpks4* (Bcin11g02700) and *Bcnrps8* (Bcin11g02650), core members of a putative secondary metabolite gene cluster on chromosome (Chr) 11 were activated during an incompatible interaction in LYC4 (**Figure 8**).

Putative gene cluster on Chr11

Accession number	Gene ID	Pfam annotation	Fold change (log2) P-value <0.05						Normalized Counts								
			BcLI10/BcMI10			BcLI10/BcLI50			BcMI10			BcLI10			BcLI50		
			12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Bcin11g02730	Bcpio5	HAD-hydrolase-like	n.s.	n.s.	3.0	n.s.	n.s.	n.s.	988	714	78	1053	1824	607	1875	3126	1244
Bcin11g02720		Aldo/keto reductase family	n.s.	n.s.	2.5	n.s.	n.s.	n.s.	600	654	97	688	1291	548	1176	1995	964
Bcin11g02710	Bcsgt2	Homodimerisation domain of SGTA	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	705	686	569	701	749	595	683	837	862
Bcin11g02700	Bcpks4	Ketoacyl-synthetase	n.s.	n.s.	4.4	n.s.	n.s.	2.6	1	2	2	6	1	53	1	3	9
Bcin11g02690		Serine hydrolase (FSH1)	n.s.	n.s.	2.3	n.s.	n.s.	n.s.	0	0	1	0	4	6	0	0	0
Bcin11g02680		AMP-binding enzyme	n.s.	n.s.	4.3	n.s.	n.s.	4.8	0	1	1	0	0	13	0	0	0
Bcin11g02670		Enoyl-(Acyl carrier protein) reductase	n.s.	n.s.	3.2	n.s.	n.s.	2.7	5	3	18	2	4	154	1	2	23
Bcin11g02660		Methyltransferase domain	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	2	5	1	2	73	0	2	4
Bcin11g02650	Bcnrps8	Condensation domain	n.s.	n.s.	3.7	n.s.	n.s.	3.2	0	0	1	1	1	16	4	0	2
Bcin11g02640		Flavin-binding monooxygenase-like	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	2	0	0	1	1	2	0	0	3
Bcin11g02630		Phytanoyl-CoA dioxygenase (PhyH)	n.s.	n.s.	3.2	n.s.	n.s.	n.s.	7	4	4	3	8	34	2	7	23



Normalized Counts: 0 1000 2000 3000

Figure 8. Expression profile of putative secondary metabolite gene cluster on Chr 11 in 10 mM sucrose LYC4 infection at 24 hpi.

Indications for stress responses in *B. cinerea* in early stages of LYC4 infection

Expression of *B. cinerea* genes involved in tolerance to antifungal compounds was substantially higher during LYC4 infection than on MM especially at early time points (Figure 9A). For instance, three ABC transporter genes *BcatrB* (Bcin13g00710), *BcatrD* (Bcin13g02720) and *Bmr3* (Bcin07g02220) with roles in the efflux of toxic compounds were highly expressed at 12 hpi on LYC4 but not on MM. Besides, *Bclcc2* encoding an extracellular laccase known to oxidize the grape phytoalexin resveratrol, was barely expressed on MM at any time point analyzed, but highly expressed during infection of LYC4 in both sucrose concentrations (**Figure 9A**).

Moreover, the expression of *BcatrB* and *Bclcc2* as well as Bcin14g05410 (encoding a transmembrane RTA1-like protein) markedly decreased after 16 h on LYC4 only during an incompatible interaction (10 mM sucrose) but not in a compatible interaction (50 mM sucrose). The differences in read count values for these three genes between 16 hpi and 24 hpi were around 2-fold for *RTA1* gene (Bcin14g05410), 4-fold for *BcatrB* and even 19-fold for *Bclcc2.*

A

Detoxification of putative toxic compounds

Accession number	Gene ID	Pfam annotation	Fold change Log(2) P-value <0.05						Normalized Counts								
			BcLI10/BcMI10			BcLI10/BcLI50			BcMI10			BcLI10			BcLI50		
			12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Bcin09g06690	-	Major Facilitator Superfamily	n.s	1.2	1.3	n.s.	n.s.	n.s.	21	57	47	33	127	112	33	130	84
Bcin07g02220	Bmr3	ABC transporter	3.2	3.6	2.1	n.s.	n.s.	n.s.	174	128	134	1600	1505	575	1964	1668	1092
Bcin13g02720	BcatrD	ABC transporter	3.2	3.1	2.0	n.s.	n.s.	n.s.	307	431	1023	2812	3619	3987	2924	3287	2957
Bcin13g00710	BcatrB	ABC transporter	3.3	2.6	n.s.	n.s.	n.s.	-2.1	252	721	1152	2517	4351	815	3123	4436	3467
Bcin14g02510	Bclcc2	Multicopper oxidase	6.3	5.3	n.s.	n.s.	n.s.	-4.3	8	28	20	713	1077	124	917	663	2363
Bcin01g00800	Bclcc8	Multicopper oxidase	n.s.	1.7	1.3	n.s.	n.s.	n.s.	313	73	64	516	246	154	409	240	158
Bcin02g02780	Bclcc13	Multicopper oxidase	n.s.	2.5	1.4	n.s.	n.s.	n.s.	345	84	33	425	494	89	486	399	187
Bcin12g05690	Bcgst20	Glutathione S-transferase	2.6	1.1	n.s.	n.s.	n.s.	n.s.	64	196	605	402	431	445	413	400	782
Bcin14g05410	-	RTA1 like protein	5.0	4.6	2.5	n.s.	n.s.	-1.1	10	26	21	307	624	115	415	581	245

Normalized Counts: 1000 2000 3000 4000

B

Detoxification of α-tomatine

Accession number	Pfam annotation	Fold Change(log2) P-value <0.05						Normalized Counts								
		BcLI10/BcMI10			BcLI10/BcLI50			BcMI10			BcLI10			BcLI50		
		12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Bcin06g00470	RTA1 like protein	n.s.	-5.2	n.s.	n.s.	n.s.	n.s.	11	118	184	1	3	73	2	6	123
Bcin09g00800	RTA1 like protein	-10.2	-4.9	n.s.	n.s.	n.s.	n.s.	175	1414	4128	17	46	1225	20	52	779
Bcin08g00070	Glycosyltransferase family 28	n.s.	-3.1	-1.7	n.s.	n.s.	n.s.	101	710	1932	38	84	595	33	81	326
Bcin08g00060	Glycosyl hydrolases family 43	n.s.	-3.7	n.s.	n.s.	n.s.	n.s.	99	966	2992	35	76	867	35	76	714
Bcin01g07660	ABC transporter	n.s.	-3.3	n.s.	n.s.	n.s.	n.s.	8	98	384	1	9	183	2	6	80
Bcin09g02710	Glycosyltransferase family 28	n.s.	-4.1	n.s.	n.s.	n.s.	n.s.	10	41	33	0	2	11	3	5	6
Bcin06g06730	hypothetical protein	n.s.	-3.1	n.s.	n.s.	n.s.	n.s.	43	238	292	28	29	123	27	26	105
Bcin09g02720	Bacterial alpha-L-rhamnosidase	n.s.	-4.5	n.s.	n.s.	n.s.	n.s.	1	21	11	2	1	5	0	2	4
Bcin02g05800	NmrA-like family	n.s.	-3.2	-2.5	n.s.	n.s.	n.s.	35	85	107	18	9	18	8	14	51
Bcin05g02090	RTA1 like protein	n.s.	-2.1	n.s.	n.s.	n.s.	n.s.	36	123	86	32	27	85	16	42	57

Normalized Counts: 0 1000 2000 3000 4000

Figure 9. Expression profile of *B. cinerea* genes related to tolerance to fungitoxic compounds. Expression levels during infection of different types of genes potentially involved in mitigating the toxicity of unknown antifungal compounds *in S. habrochaites* (A); Expression of α-tomatine-responsive genes (B).

Delayed induction of α-tomatine-responsive genes during LYC4 infection as compared to MM

The *in vitro* transcriptional induction of *B. cinerea* genes by the tomato saponin α-tomatine was shown to be an important response in the mitigation of its antifungal effects (**Chapter V**). These α-tomatine-responsive genes were also up-regulated during tomato infection and are important for virulence in tomato (**Chapter V**). In this study, α-tomatine-responsive genes encoding the tomatinase BcTom1 (Bcin08g00060), an ABC transporter (Bcin01g07660), a glycosyltransferase from the GT28 family (Bcin08g00070) and several RTA1 membrane proteins (Bcin06g00470, Bcin09g00800 and Bcin05g02090) were all 7- to 10-fold induced at 16 h after inoculation on MM. By contrast, the induction of these genes was only observed at 24 hpi on LYC4, manifested as significantly lower expression levels in BcLI1016 and BcLI5016 in comparison to BcMI1016 (**Figure 9B**).

Differential expression of fungal genes associated with plant cell death induction

Induction of plant cell death is an important characteristic of *B. cinerea* that enables the fungus to cause disease. Therefore, we also investigated the genes encoding proteins that were reported to induce plant cell death or enzymes involved in the biosynthesis of the phytotoxins botrydial and botcinic acid as summarized in (Leisen et al., 2022).

In general, most of these genes were expressed at high levels during LYC4 and MM infection, although some of them displayed different expression patterns (**Figure 10**). Genes including *Bcpg2* (Bcin14g00610), *Bcnep1* (Bcin06g06720) and the botrydial biosynthetic cluster (Bcin12g06370-Bcin12g06430), exhibited increased expression in early time points with transcription peaking at 16 hpi and then decreased at 24 hpi. By contrast, continuous increase of gene expression in the first 24 h was observed for genes such as *Bcnep2* (Bcin02g07770) and *Bcpg1* (Bcin14g00850). Interestingly, certain genes displayed different expression patterns in MM and LYC4, or in distinct inoculation conditions (10 or 50 mM sucrose). For instance the expression of the botrydial gene cluster continued to increase in LYC4 (inoculated with 50 mM sucrose) even at 24 hpi which led to significantly higher expression in BcLI5024 than in BcLI1024 and BcMI1024.

The transcript levels of most of the genes associated with induction of plant cell death were higher in MM than in LYC4 (both with 10 mM sucrose) with some exceptions. For instance, *Bcieb1* (Bcin15g00100), *Bcspl1* (Bcin03g00500) and *Bccdi1* (Bcin06g00550) exhibited higher expression in LYC4 than in MM after 10 mM sucrose inoculation.

Expression of genes in *B. cinerea* mini-chromosomes during tomato infection

The genome of B05.10 contains 18 chromosomes of which Chr17 and Chr18 are small (250 kb and 210 kb in length, respectively), seemingly unstable and thereby considered as accessory chromosomes (van Kan et al., 2017). Multiple genes on Chr17 and Chr18 were expressed during tomato infection (**Supplementary Figure S1**). Moreover, a putative flavin oxidoreductase-encoding gene on Chr18 (Bcin18g00020) displayed high transcript levels at 16 hpi on MM as compared to LYC4 infection (**Supplementary Figure S1**).

LYC4 and MM strongly differed in transcriptional profiles in absence of *B. cinerea*

LYC4 and MM leaf samples displayed high numbers of DEGs even upon mock treatment (**Figure 11A**). For instance, prior to *B. cinerea* infection, in total 8240 tomato genes in LYC4 were differentially expressed as compared with MM (**Figure 11A**). The high number of DEGs further increased after *B. cinerea* inoculation (**Figure 11B**).

Induction of plant cell death

Accession number	Gene ID	Pfam annotation	Fold Change(log2) P-value <0.05 BcLI10/BcMI10			BcLI10/BcLI50			Normalized Counts BcMI10			BcLI10			BcLI50		
			12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Botrydial biosynthesis gene cluster																	
Bcin12g06430	Bcbot7	short chain dehydrogenase	-2.4	-1.3	n.s.	n.s.	n.s.	-2.7	3655	16387	2117	712	6865	3765	647	9334	23821
Bcin12g06420	Bcbot6	Fungal Zn(2)-Cys(6) binuclear cluster domain	n.s.	-1.1	n.s.	n.s.	n.s.	-1.9	211	803	288	73	368	240	66	463	901
Bcin12g06410	Bcbot5	Transferase family	n.s.	-1.3	n.s.	n.s.	n.s.	-2.2	1032	5107	1182	276	2131	1528	171	2517	6936
Bcin12g06400	Bcbot3	Cytochrome P450	-2.1	-1.1	n.s.	n.s.	n.s.	-1.8	1753	7158	1792	418	3242	2839	324	3765	10037
Bcin12g06390	Bcbot2	-	n.s.	-1.4	n.s.	n.s.	n.s.	-2.3	2400	11902	2542	577	4567	2980	439	5280	14620
Bcin12g06380	Bcbot1	Cytochrome P450	-1.9	-1.3	n.s.	n.s.	n.s.	-2.0	2974	14681	3820	794	6034	5475	568	7060	22200
Bcin12g06370	Bcbot4	Cytochrome P450	-1.9	-1.3	n.s.	n.s.	n.s.	-2.2	1654	7888	1614	436	3117	2035	324	3651	9517
Botcinic acid biosynthesis gene cluster																	
Bcin01g00160	Bcboa17	short chain dehydrogenase	n.s.	n.s.	-2.0	n.s.	n.s.	-0.9	102	287	1219	33	132	314	33	81	578
Bcin01g00150	Bcboa16	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	0	0	0	2	0	0	0	1
Bcin01g00140	Bcboa15	Fungal specific transcription factor domain	n.s.	n.s.	n.s.	n.s.	n.s.	1.3	39	47	84	32	43	91	23	27	36
Bcin01g00130	Bcboa13	Fungal Zn(2)-Cys(6) binuclear cluster domain	n.s.	-0.8	n.s.	n.s.	n.s.	n.s.	151	601	600	97	341	445	68	251	485
Bcin01g00120	Bcboa12	-	n.s.	n.s.	-1.2	n.s.	n.s.	n.s.	254	1566	1357	97	730	605	47	451	489
Bcin01g00110	Bcboa11	Transferase family	n.s.	n.s.	-1.3	n.s.	n.s.	n.s.	161	812	893	80	390	350	27	271	350
Bcin01g00100	Bcboa10	Thioesterase domain	n.s.	n.s.	-1.9	n.s.	n.s.	n.s.	234	1353	1672	78	446	454	41	313	508
Bcin01g00090	Bcboa9	Phosphopantetheine attachment site	n.s.	n.s.	-0.8	n.s.	n.s.	n.s.	1017	4902	5620	392	2292	3283	186	1652	1832
Bcin01g00080	Bcboa8	FAD binding domain	n.s.	-1.1	n.s.	n.s.	n.s.	n.s.	254	1246	1089	115	570	750	99	400	720
Bcin01g00070	Bcboa7	Cytochrome P450	n.s.	n.s.	-1.0	n.s.	n.s.	n.s.	500	3261	3435	286	1563	1670	220	1037	1365
Bcin01g00060	Bcboa6	Beta-ketoacyl synthase	n.s.	n.s.	-0.9	n.s.	n.s.	n.s.	884	4202	4648	285	1854	2436	164	1236	1797
Bcin01g00050	Bcboa5	Alcohol dehydrogenase GroES-like domain	n.s.	n.s.	-1.4	n.s.	n.s.	n.s.	162	1058	938	72	431	364	30	293	383
Bcin01g00040	Bcboa4	Cytochrome P450	n.s.	n.s.	-1.1	n.s.	n.s.	n.s.	352	2454	2490	134	960	1174	74	630	942
Bcin01g00030	Bcboa3	Cytochrome P450	n.s.	n.s.	-0.9	n.s.	n.s.	n.s.	674	4962	5440	256	2000	2839	132	1299	1932
Bcin01g00020	Bcboa2	Flavin-binding monooxygenase-like	n.s.	n.s.	-1.1	n.s.	n.s.	n.s.	147	955	979	74	489	471	44	323	420
Bcin01g00010	Bcboa1	NmrA-like family	n.s.	n.s.	-1.3	n.s.	n.s.	n.s.	788	5077	4738	328	2442	1941	201	1669	2292
Cell wall degrading enzymes																	
Bcin03g00480	Bcxyn11A	Glycosyl hydrolases family 11	n.s.	-1.0	-1.7	n.s.	n.s.	1.1	95	390	4587	56	191	1361	58	174	615
Bcin03g03630	Bcxyg1	Glycosyl hydrolase family 12	n.s.	n.s.	-1.4	n.s.	n.s.	1.2	3776	8098	12735	3106	6309	4696	3091	5108	2086
Bcin09g01800	Bcxyl1	GDSL-like Lipase/Acylhydrolase family	n.s.	n.s.	n.s.	n.s.	n.s.	1.1	21	11	17	21	7	36	12	22	16
Bcin04g04190	Bcgs1	Starch binding domain	n.s.	n.s.	n.s.	n.s.	n.s.	-2.0	679	4932	838	403	5237	1859	380	4752	7179
Bcin14g00610	Bcpg2	Glycosyl hydrolases family 28	n.s.	n.s.	-0.8	n.s.	n.s.	-1.0	6572	18286	7667	8331	26906	4372	8340	21334	8507
Bcin14g00850	Bcpg1	Glycosyl hydrolases family 28	n.s.	0.8	-0.8	n.s.	n.s.	-0.7	8880	15006	38478	10781	25727	22527	11817	24326	36462
Bcin02g05860	Bcpg6	Glycosyl hydrolases family 28	n.s.	-1.8	-1.7	n.s.	n.s.	3.2	522	2933	16130	300	834	5033	210	703	560
Bcin03g01680	Bcpg4	Glycosyl hydrolases family 28	n.s.	n.s.	-1.9	n.s.	n.s.	3.9	14	120	8242	10	21	2183	17	22	142
Othter Necrosis and ethylene-inducing protein																	
Bcin06g06720	Bcnep1	Necrosis inducing protein (NPP1)	n.s.	n.s.	-1.9	n.s.	n.s.	n.s.	8106	13159	2952	5836	12067	771	5587	11904	487
Bcin02g07770	Bcnep2	Necrosis inducing protein (NPP1)	n.s.	-2.7	-2.7	n.s.	n.s.	n.s.	39	250	2696	13	37	423	11	32	313
Bcin14g01200	Bchip1	-	n.s.	1.5	n.s.	n.s.	n.s.	n.s.	494	686	1312	862	1957	1742	902	1920	1666
Bcin10g01020	Bcplp1	-	n.s.	-1.6	-1.4	n.s.	n.s.	-1.7	525	1004	451	294	337	167	168	316	529
Bcin15g00100	Bcieb1	-	1.2	2.0	1.0	n.s.	n.s.	n.s.	429	359	466	990	1488	915	1291	1417	1109
Bcin03g00500	Bcspl1	Cerato-platanin	n.s.	1.2	1.9	n.s.	n.s.	3.0	83	127	715	207	286	2619	145	271	330
Bcin01g06010	Bccrh1	Glycosyl hydrolases family 16	n.s.	-1.5	n.s.	n.s.	n.s.	n.s.	193	355	311	152	119	334	146	138	304
Bcin05g03680	BcSsp2	-	n.s.	-2.9	-5.3	n.s.	n.s.	-2.3	85	831	1351	52	110	34	30	88	167
Bcin06g00550	BcCDl1	-	n.s.	1.6	2.8	n.s.	n.s.	3.0	50	22	32	43	63	221	58	86	28

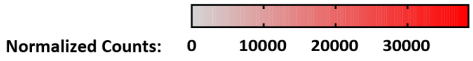


Figure 10. Expression of *B. cinerea* genes associated with plant cell death induction.

Delayed and attenuated transcriptional response to *B. cinerea* infection in LYC4

B. cinerea elicited transcriptional changes both in MM and LYC4, however, to very different extent in terms of the number of DEGs. MM displayed 4216 up-regulated genes and 4027 down-regulated genes (as compared with mock treatment) at 12 hpi and the numbers further increased to 6291 and 6210, respectively, at 24 hpi (**Figure 12A**). By contrast, transcriptional reprogramming was much less pronounced in LYC4 in comparison with MM. For instance, only 211 up-regulated genes and 65 down-regulated genes were observed in 10 mM sucrose infection on LYC4 at 12 hpi. Moreover, 2080 out of the 3609 (SolycLI1024) and 3596 (SolycLI5024) genes that were up-regulated in LYC4 at 24 hpi, were already found to be up-regulated at 12 hpi during MM infection. This indicates a delayed transcriptional response in LYC4 to *B. cinerea* infection as compared to MM (**Figure 12B**).

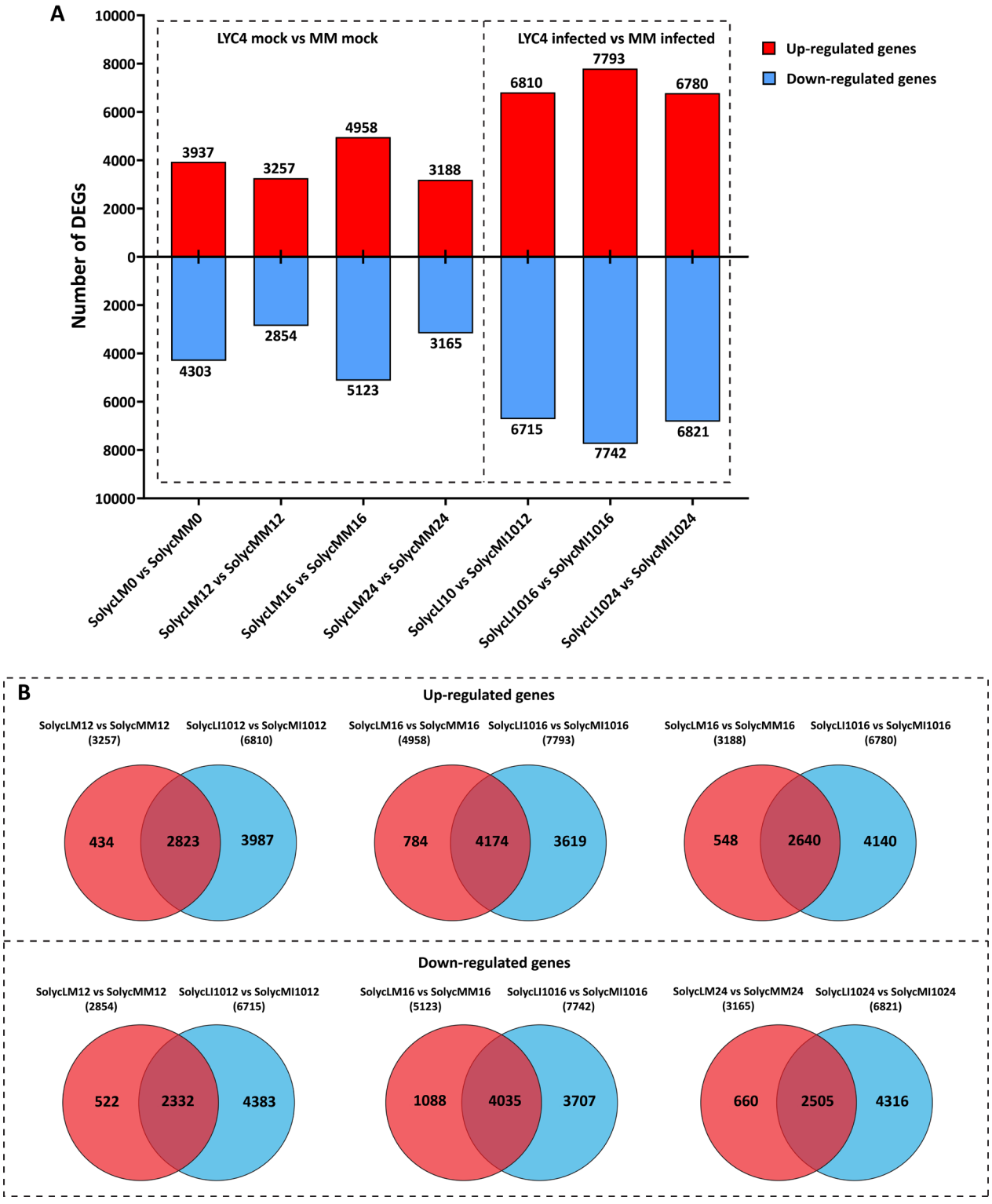


Figure 11. Number of plant DEGs between LYC4 and MM both in the mock and infected samples (A) and common plant DEGs between comparison of LYC4 and MM mock samples and comparison of LYC4 and MM infected samples (B).

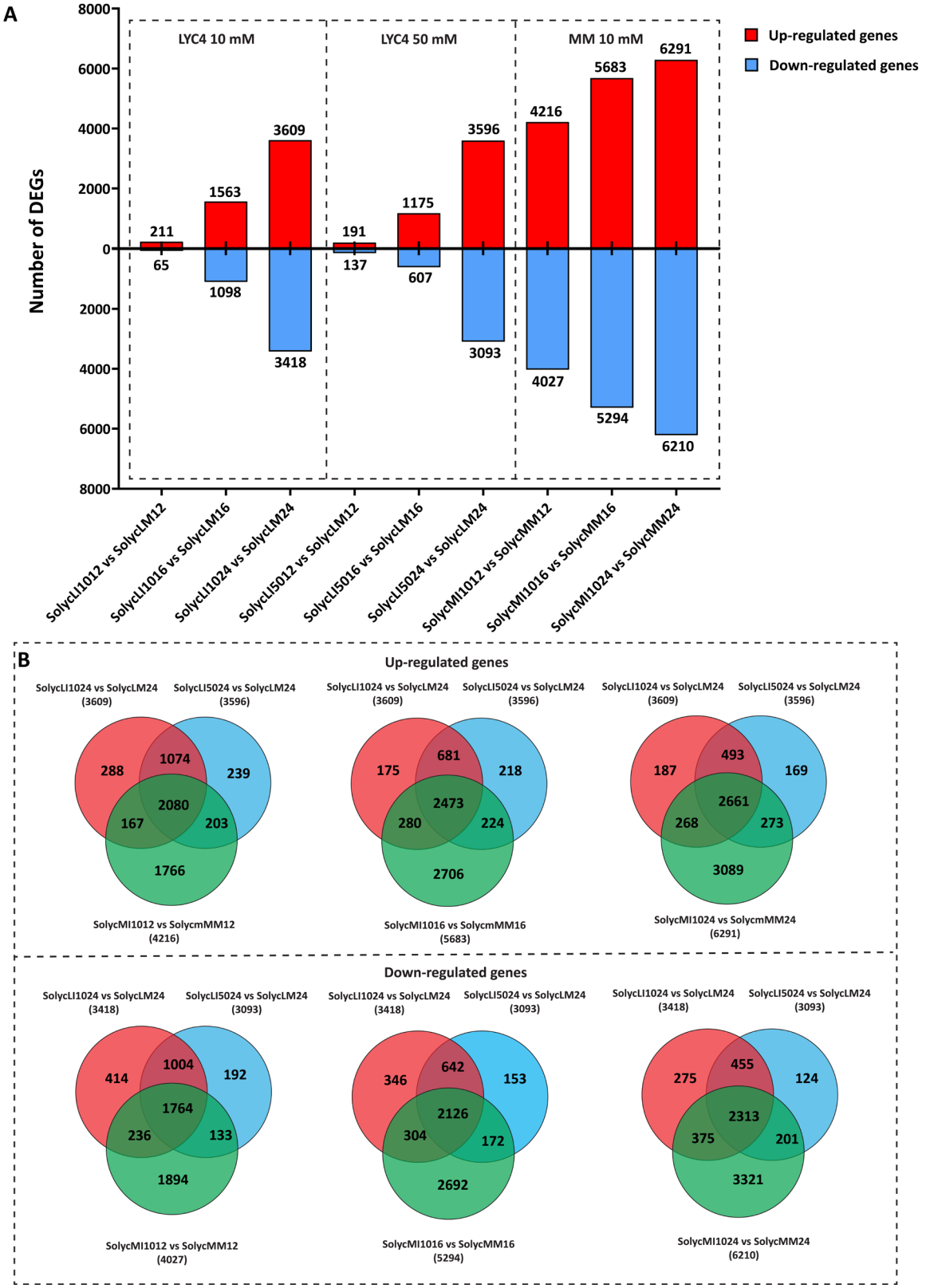


Figure 12. Number of plant DEGs after *B. cinerea* inoculation as compared with mock treatment (A) and common plant DEGs between LYC4 infection and MM infection (B).

Discussion

The expression pattern of a large number of *B. cinerea* genes associated with usage of carbohydrates including glycosyl hydrolases and sugar transporters was typified by low transcript levels at 12 and 16 hpi, followed by up-regulation at 24 hpi but only in 10 mM sucrose (both during plant infection and *in vitro* growth). This expression profile can be explained by the occurrence of carbon catabolite repression (CCR) at early time points, when sucrose is plentiful. At later time points, repression is relieved as a result of the depletion of sucrose by fungal growth, however, CCR relief was only achieved within the time frame of the experiment when the initial sucrose concentration was 10 mM, and not at 50 mM. CCR is a well-known mechanism which represses the expression of genes encoding enzymes for utilizing less-favoured carbon sources when an optimal carbon source like glucose or sucrose is present (Fasoyin et al., 2018). The slight up-regulation of autophagy-related genes at the same time (**Supplementary Figure S2**) in the 10 mM sucrose infection (BcLI1024 and BcMI1024) suggested that the fungus might be at the onset of starvation at 24 hpi (Khan et al., 2012). Besides, the up-regulation of a catabolic pathway to utilize D-galacturonic acid exclusively in the 10 mM sucrose infection at 24 hpi implied that the fungus was specifically consuming pectin-derived carbon sources released from plant cell wall degradation to substitute for the depleted sucrose provided in the inoculum.

Up-regulation of *B. cinerea* genes encoding secreted proteins such as glycosyl hydrolases might also result in elicitation of host defense responses and plant cell death. Several CWDEs have been reported to be capable of causing cell death in different hosts (Nakajima and Akutsu, 2014). Hypothetically, the confinement of *B. cinerea* infection on LYC4 during a incompatible interaction might be partially attributed to the LYC4 repertoire of receptors that perceive these proteins and execute the subsequent cell death process. It has been reported that wild tomato species such as *S. pimpinellifolium* and *S. habrochaites* harbor unique receptor-like proteins (RLPs) that can recognize extracellular proteins from *Cladosporium fulvum* and initiate host cell death referred to as hypersensitive response (HR) (Kruijt et al., 2005; Takken et al., 1999; Thomas et al., 1997).

A striking, unique feature in the transcriptional profile of the incompatible interaction at 24 hpi (BcLI1024) was the simultaneous down-regulation of a large number of genes encoding ribosomal proteins, ribosomal RNA processing energy generation. Such down-regulation was not observed in at earlier timepoints of the same interaction, nor in any of the compatible interactions (BcLI50, BcMI10). This observation provides strong evidence for the stress situation that *B. cinerea* was experiencing, resulting in shutting down ribosome assembly, protein translation and other vital functions.

An interesting observation is that the induction of α-tomatine-responsive genes in *B. cinerea* identified in *in vitro* assay (**Chapter V**) was slower during LYC4 infection than in MM. α-Tomatine is mainly stored in large quantities in the vacuoles of tomato cells and can be released into the apoplast after the cell is damaged (Kazachkova et al., 2021). Although it can be secreted from the tomato roots to the rhizosphere, there is no indication that α-tomatine is actively secreted from cytoplasm to the extracellular space upon pathogen attack in the leaf (Nakayasu et al., 2020). Therefore, the abrupt high expression of α-tomatine-responsive genes is in response to the release of α-tomatine from host cells undergoing massive cell death in the primary lesion. The abrupt induction of α-tomatine-responsive genes occurred at 16 and 24 hpi during MM infection, however, in LYC4, the induction was only observed at 24 hpi and the response was not as strong as in MM. The late induction of α-tomatine-responsive genes during LYC4 infection, as compared to MM infection, might be a consequence of a significantly delayed plant cell death induction in LYC4. We performed various histochemical staining experiments to get insight into the timing and modes of cell death induction in LYC4, however, these experiments have largely remained inconclusive.

Membrane transporters such as ABC transporters (BcatrB, BcatrD and Bmr3) and MFS (Bcmfs1) are well-characterized efflux pumps in *B. cinerea* whose expression can be specifically induced by toxic plant compounds and artificial fungicides. For instance, the expression of *BcatrB* can be up-regulated in the presence of camptothecin, eugenol, psoralen, resveratrol and rishitin (Schoonbeek et al., 2003) and the capacity to induce *BcatrB* expression is thought to be confined to substrates of the transporter. Therefore, expression analyses of ABC transporter genes can provide information about their putative substrates. Instead of exporting the toxic compounds, fungi also employ another defense strategy which involves the enzymatic conversion of the antifungal compounds into less toxic substances. Bclcc2 is an extracellular laccase that can oxidize plant phenolic compounds such as resveratrol and tannins both *in vitro* and during plant infection (Schouten et al., 2002). The inducers of *Bclcc2* expression not only comprise phenolics but include cupric ions such as $CuSO_4$ (Buddhika et al., 2021). Therefore, the high expression of genes involved in the defense against toxic compounds during LYC4 infection indicates that the fungus was under stress, presumably related to plant antimicrobial secondary metabolites that are either unique to LYC4 or that accumulate at higher concentrations on LYC4 as compared to MM. Moreover, the strong transcriptional response in *B. cinerea* during the interaction with LYC4 in the early stages (especially at 12 hpi) was prior to host cell death after penetration. This observation suggests that the putative toxic metabolites that trigger this response might be located on the surface of LYC4 leaves. *S. habrochaites* is well-known to produce specialized antimicrobial secondary metabolites from the glandular trichomes (Fan et al., 2019). Several sesquiterpenes (Sallaud et a., 2009; Therezan et al., 2021; Wang et al., 2020;), flavonoids (Kim et

al., 2014; Schmidt et al., 2011), and acylated sugars (Ghosh et al., 2014) are either uniquely accumulated in the trichomes of *S. habrochaites* or present in higher quantity in *S. habarochiates* than in the cultivated tomato *S. lycopersicum*.

The high expression of the membrane transporter genes and *Bclcc2* was also observed in the LYC4 infection using 50 mM sucrose medium highlighting that this phenomenon was specific to *S. habrochaites*, and not necessarily to the (compatible or incompatible) interaction. maybe reflecting the presence of substrates for the transporters and the laccase in LYC4, but not in MM. Whether the activation of these genes especially in early stages of the interaction can impact later processes in the infection needs to be studied.

The incompatible interaction was manifested as tiny, dispersed black lesions within the droplet area indicating that the fungus was unable to further establish biomass and cause expanding lesions. Some fungal genes related to the induction of plant cell death were only slightly lower expressed at 24 hpi during incompatible interaction (BcLI1024) than in the compatible interactions at the same time point (BcLI5024 and BcMI1024) which suggests that failure of *B. cinerea* to expand beyond the inoculation spot was not due to the loss of plant cell death inducing capacity. However, the expression of genes associated with translation (ribosome proteins) and transcription (mRNA) as well as the energy generation (ATP synthase) at 24 hpi exhibited higher expression levels during compatible interaction (BcLMI1024 and BcLI5024) than during incompatible interaction (BcLI1024). This result implied that, in the incompatible interaction, primary house-keeping functions in *B. cinerea* were arrested and the fungus was compromised in its growth. Overall, during LYC4 infection, the delayed induction of plant cell death at later time points and the high expression of detoxification-related fungal genes in the early stages collectively imply that LYC4 possesses mechanisms that can slow down or sabotage the fungal infection process in the first 24 h.

Materials and methods

Plant infection

The growth of tomato plants and fungal pathogen *B. cinerea* was described in **Chapter II**. *B. cinerea* spore suspensions in GB5-10 mM/50 mM sucrose media at 1000 spores/µL were inoculated on the adaxial sides of detached tomato leaves under the lab conditions. Leaf discs containing 5 inoculation droplets (2 µL per droplet) were sampled at 0 hpi, 12 hpi, 16 hpi and 24 hpi, respectively, with three biological replicates for each condition. *In vitro* grown B05.10 liquid cultures were collected at the same time points with two biological replicates. All the samples were immediately frozen in liquid nitrogen after sampling and then freeze dried. mRNA extraction was performed using Maxwell 16 LEV Plant RNA Kit (Promega). Illumina RNA-seq was carried out at carried out at Beijing Genomics Institute (BGI), Shenzhen, China.

Gene expression analysis

The RNA sequencing reads were mapped to the publicly available genomes of *B. cinerea* strain B05.10 and *S. lycopersicum* (version SL4.0) using STAR (Dobin et al.,2013). Subsequently, read counting for each gene was achieved by using HTSEQ-count (Anders et al., 2015). Isoforms of *B. cinerea* with 0 read counts in all samples were removed.

Analysis of differentially expressed genes (DEGs)

DEG analysis in this study was performed by Bioconductor R package DESeq2, version 1.30.1 (Love et al., 2014). The whole analysis includes sample clustering analysis, principal component analysis and multiple testing analysis. The prepared merged count files in the form of a matrix of non-normalized counts, were used as quantification input files for DESeq2. Before analysis, genes with less than 10 counts for all samples were filtered out to make sure only informative genes were included. The DESeqDataSet object (dds), was constructed by function DESeqDataSetFromMatrix() (Love et al., 2014).

To accurately compare gene expression levels between different biological conditions, read counts of genes were normalized. DESeq2 built-in function estimateSizeFactors() was applied on the dds object to correct for sequencing depth and RNA composition (Love et al., 2014). Later, counts() function was applied on normalized dds to extract normalized counts data.

Gene ontology enrichment analysis for *B. cinerea* genes

Gene ontology (GO) enrichment analysis for *B. cinerea* was performed with the R package AnnotationHub (version 2.22.1) (Martin and Lori, 2021) and clusterProfiler (version 3.18.1) (Yu et al., 2012) using Entrez IDs of *B. cinerea* genes. Cutoff p-value and q-value was set at 0.05. Ontologies used in our study included biological process (BP) and molecular function (MF).

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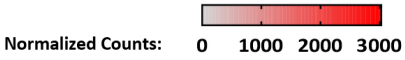
Supplementary data

Mini-chromosomes

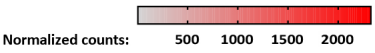
Accession number	Pfam annotation	Fold change (log2) P-value <0.05						Normalized Counts								
		BcLI10/BcMI10			BcLI10/BcLI50			BcMI10			BcLI10			BcLI50		
		12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Chr18																
Bcin18g00190	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	13	27	40	11	39	38	16	18	27
Bcin18g00180	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	7	10	5	4	9	9	8	7	13
Bcin18g00170	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin18g00160	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	2	1	0	0	3	1	2	1
Bcin18g00150	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	11	30	21	6	25	9	8	19	12
Bcin18g00145	-	n.s.	n.s.	n.s.	n.s.	n.s.	0.8	128	147	137	82	154	203	79	114	118
Bcin18g00140	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	2	3	3	1	0	7	2	3	4
Bcin18g00135	-	n.s.	n.s.	n.s.	n.s.	n.s.	2.1	0	4	10	0	2	15	1	1	3
Bcin18g00130	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin18g00120	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	1	0	0	0	0	0	0
Bcin18g00110	-	n.s.	n.s.	n.s.	n.s.	n.s.	-0.7	33	55	36	36	79	30	28	65	48
Bcin18g00060	Ulp1 protease family	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	30	62	48	47	72	58	44	62	44
Bcin18g00040	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	1	1	2	0	2	0
Bcin18g00020	NADH: flavin oxidoreductase	-1.2	-2.0	-1.7	n.s.	n.s.	-2.9	501	3022	404	220	752	126	240	839	926
Bcin18g00010	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	1	9	4	2	8	8	3	7	12
Chr17																
Bcin17g00230	Ulp1 protease family	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	48	63	36	55	63	41	45	52	50
Bcin17g00180	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin17g00170	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	1	1	1	0	1	0	0	2
Bcin17g00160	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin17g00150	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin17g00140	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	20	36	38	26	48	48	19	38	39
Bcin17g00130	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	3	2	1	3	4	5	1	3	3
Bcin17g00120	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	1	0	0	0	0	0
Bcin17g00110	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	1	1	1	0	1	0	0	0
Bcin17g00090	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	0	0	0	0
Bcin17g00070	Ulp1 protease family	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	75	128	114	69	113	108	70	117	116
Bcin17g00065	-	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	52	91	66	41	66	50	35	74	59
Bcin17g00060	Protein kinase domain	n.s.	n.s.	n.s.	n.s.	n.s.	1.5	15	12	10	19	18	25	13	9	9
Bcin17g00050	Phosphopantetheine attachment site	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0	0	0	0	0	1	2	0	2
Bcin17g00040	Alpha/beta hydrolase family	4.9	n.s.	n.s.	n.s.	n.s.	n.s.	2	4	19	47	14	97	46	13	153
Bcin17g00030	-	n.s.	n.s.	1.9	n.s.	n.s.	n.s.	2	7	12	4	10	46	10	16	52
Bcin17g00020	UbiA prenyltransferase family	n.s.	n.s.	n.s.	n.s.	n.s.	-2.1	0	0	1	1	1	4	2	1	15
Bcin17g00010	Aminotransferase class I and II	n.s.	n.s.	n.s.	n.s.	n.s.	-2.0	1	1	1	4	4	8	5	4	29



Supplementary Figure S1. Expression profile of *B. cinerea* genes on mini-chromosomes Chr17 and Chr18 during tomato infection.

Autophagy

Accession number	Gene ID	Pfam annotation	Fold change (log2) P-value <0.05						Normalized counts								
			BcLI10/BcMI10			BcLI10/BcLI50			BcMI10			BcLI10			BcLI50		
			12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI	12 HPI	16 HPI	24 HPI
Bcin14g01550	Bcatg2	Autophagy-related protein	n.s.	n.s.	0.6	n.s.	n.s.	1.4	76	103	247	111	128	365	83	141	138
Bcin08g04530	Bcatg3	Autophagocytosis associated protein	n.s.	n.s.	0.6	n.s.	n.s.	1.1	113	137	223	181	191	332	159	166	156
Bcin09g05260	Bcatg5	Autophagy protein Apg5	n.s.	n.s.	n.s.	n.s.	n.s.	0.9	42	53	83	37	89	84	44	64	44
Bcin09g04730	Bcatg7	Ubiquitin-like modifier-activating enzyme ATG7	n.s.	n.s.	n.s.	n.s.	n.s.	0.5	72	93	159	83	122	189	86	124	129
Bcin02g02570	Bcatg8	Autophagy protein Atg8 ubiquitin like	n.s.	n.s.	0.4	n.s.	n.s.	1.1	750	887	1741	1026	1210	2325	1038	990	1081
Bcin15g00650	Bcatg9	Autophagy protein Apg9	n.s.	n.s.	0.7	n.s.	n.s.	1.2	46	71	104	69	111	164	58	67	73
Bcin11g05150	Bcatg11	Autophagy-related protein 11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	71	104	112	63	98	116	49	88	108
Bcin16g00660	Bcatg12	Ubiquitin-like autophagy protein Apg12	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	7	12	10	7	9	12	6	6	10
Bcin13g04910	Bcatg13	Autophagy-related protein 13	n.s.	n.s.	n.s.	n.s.	n.s.	0.8	171	219	344	230	256	433	183	268	249



Supplementary Figure S2. Expression profile of autophagy related genes during incompatible interactions on LYC4 at 24 hpi.

Chapter 4

Bitter and sweet make tomato hard to (b)eat

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Abstract

The glycoalkaloid saponin α-tomatine is a tomato-specific secondary metabolite that accumulates to mM levels in vegetative tissues, and has antimicrobial and antinutritional activity that kills microbial pathogens and deters herbivorous insects. We describe recent insights into the biosynthetic pathway of α-tomatine synthesis and its regulation. We discuss the mode of action of α-tomatine by physically interacting with sterols and thereby disrupting membranes, and how tomato protects itself from its toxic action. Tomato pathogenic microbes can enzymatically hydrolyse and thereby inactivate α-tomatine using either of three distinct types of glycosyl hydrolases. We also describe findings which extend well beyond the simple concept of plants producing toxins and pathogens inactivating them. There are reports that toxicity of α-tomatine is modulated by external pH; that α-tomatine can trigger programmed cell death in fungi; that cellular localization matters for the impact of α-tomatine on invading microbes; and that α-tomatine breakdown products generated by microbial hydrolytic enzymes can modulate plant immune responses. Finally we address a number of outstanding questions that deserve attention in the future.

Introduction

The conquest of South and Middle America in the 16th and 17th century resulted in the import into Europe of several food crops indigenous to the America's, including tomato and potato. These Solanaceae were soon discovered to produce bitter-tasting glycoalkaloids in vegetative organs, of which consumption could result in serious poisoning. It was later observed that, apart from their antinutritional effect, glycoalkaloids also possess antibiotic activity, are repellent or toxic to pest insects and may have useful medicinal applications. In the past decades, the tomato glycoalkaloid saponin α-tomatine has been extensively studied for its role in the interaction of plants with pest insects and pathogens, often with the aim of improving plant health. In this paper we present an overview of α-tomatine as a broad-spectrum toxic plant compound that protects tomato from herbivores and pathogens, and we discuss recent insights into its biosynthesis and regulation. Furthermore we describe how microbial pathogens cope with the inhibitory activity of α-tomatine and may even exploit the hydrolytic breakdown products of α-tomatine to modulate plant immune responses.

α-tomatine: the major tomato saponin with antibiotic activity

The study of α-tomatine started from the exploration of fungistatic agents in tomato tissues. Over 70 years ago, Fontaine et al. (1948) named the first purified compound from tomato leaves possessing antifungal property as tomatine. It was identified as a glycosidal alkaloid also known as steroidal glycoalkaloids (SGAs) which are a subgroup of saponins. Later studies revealed that its chemical structure is composed of a steroidal aglycon ("tomatidine") and a tetrasaccharide side branch (β-lycotetraose) containing two molecules of glucose and one molecule each of galactose and xylose. α-tomatine is the name of the form with the tetrasaccharide, whereas the other forms lacking a terminal xylose or terminal glucose, or both terminal sugars were designated as β_1-tomatine, β_2-tomatine and γ-tomatine, respectively (Figure 1; Kuhn et al., 1956; Kuhn et al., 1957). α-Tomatine is the major SGA as well as the main saponin in vegetative tissues and green fruits; its concentration can be up to several mM on a fresh weight basis (Iijima et al., 2013; Friedman & Levin, 1998; Keukens et al., 1995; Kozukue et al., 2004). The high α-tomatine levels in immature fruit decrease during ripening by the conversion to esculeoside A (**Figure 1**; Cárdenas et al., 2019; Iijima et al., 2009; Mintz-Oron et al., 2008; Nakayasu et al., 2020). The antimicrobial activity of α-tomatine was first demonstrated on the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Roddick, 1974), thereafter antibiotic property was reported against many tomato pathogens (including fungi, oomycetes and bacteria), as well as pest insects (Altesor et al., 2014; Campbell & Duffey, 1979; Chowánski et al., 2016; Kaup et al., 2005; Seipke & Loria, 2008; Sandrock & VanEtten., 1998). Because of its high

concentration and broad-spectrum of *in vitro* antibiotic activity, α-tomatine has long been studied as a defense compound that might confer resistance to tomato pathogens. Here, we will discuss old and recent knowledge about the relevance and mode of action of α-tomatine, and illustrate its versatile biological properties, that extend well beyond the perception of a "simple" membrane perforating toxin.

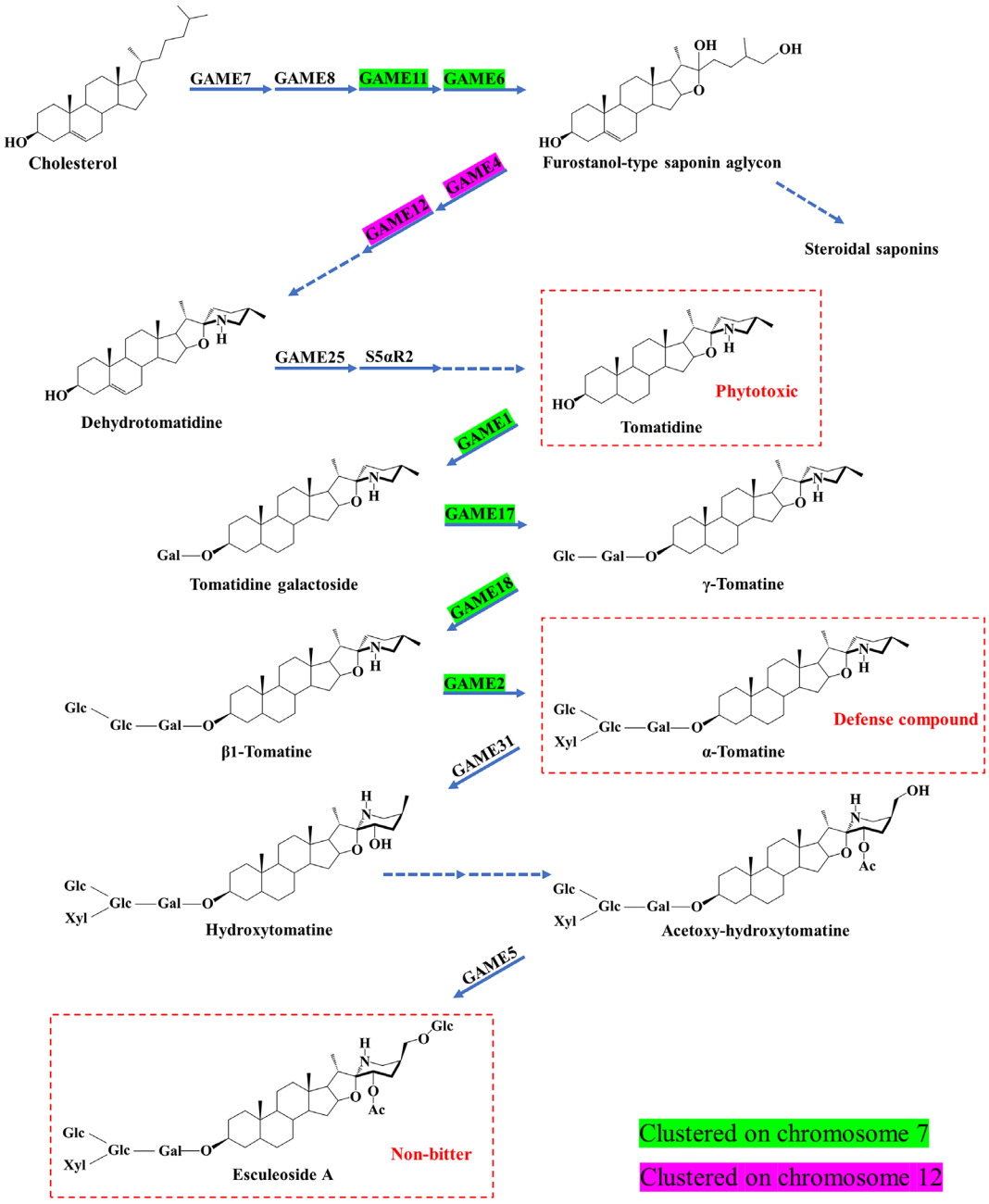

Figure 1. Biosynthetic pathway of α-tomatine from cholesterol. Chemical structures and names of several biosynthetic intermediates are provided. The arrows represent catalytic conversions, with the gene name (if characterized) provided above the arrow. Genes in coloured boxes are in close physical proximity in a genomic cluster on tomato chromosomes 7 and 12. The red stippled boxes highlight three key components: the phytotoxic precursor tomatidine, the defense compound α-tomatine and the non-bitter breakdown product esculeoside A.

The biosynthetic pathway of α-tomatine and its regulation

Although α-tomatine was identified as the major SGA in tomato more than 70 years ago, the metabolism and regulation of its synthesis are not fully understood. Initial studies on α-tomatine metabolism were driven by an interest to improve fruit quality by removing the anti-nutritional trait caused by α-tomatine. However, as a potent defense metabolite, insight into its metabolism can also help to increase α-tomatine levels in vegetative tissue and thereby contribute to resistance.

SGAs, including α-tomatine, are synthesized from cholesterol although cholesterol biosynthesis in plants itself is not fully understood. Conversion of cholesterol to α-tomatine (Figure 1) involves multiple reactions mediated by enzymes encoded by *GLYCOALKALOID METABOLISM* (*GAME*) genes (Itkin et al., 2013). The first part of the pathway requires four enzymes (GAME7, GAME8, GAME11 and GAME6) and results in the synthesis of a saponin aglycon that serves as precursor for both glycoalkaloids and steroidal saponins. The first dedicated step towards glycoalkaloid production is the oxidation of saponin aglycon by GAME4, followed by multiple additional conversions to form the aglycon alkaloid tomatidine (Itkin et al., 2013). Recent research revealed that the conversion from dehydrotomatidine to tomatidine is not mediated by a single reaction as suggested by (Friedman, 2002; Itkin et al., 2013), but rather involves multiple steps including oxidation, isomerization and reduction and requires a short-chain dehydrogenase/reductase (GAME25, also known as Sl3βHSD) and steroid 5α-reductase (SlS5αR2) (Akiyama et al., 2019; Lee et al., 2019; Sonawane et al., 2018). As shown in Figure 1, the synthesis of α-tomatine from its aglycon tomatidine requires four consecutive glycosylations by distinct glycosyltransferases GAME1, GAME17, GAME18 and GAME2 (Itkin et al., 2011; Itkin et al., 2013). During tomato fruit ripening the decrease of α-tomatine content results from conversion to esculeoside A, a non-bitter steroidal glycoalkaloid. This process involves GAME31, a 2-oxoglutarate-dependent dioxygenase (also known as Sl23DOX) that catalyzes the hydroxylation of α-tomatine and the a recently identified glycosyltransferase GAME5 which produces esculeoside A (Figure 1; Cárdenas et al., 2019; Nakayasu et al., 2020; Szymański et al., 2020).

Interestingly, six *GAME* genes are physically clustered on tomato chromosome 7 (Figure 1), including *GAME11* and *GAME6* which are required in the production of the furostanol-type saponin aglycon along with the four genes encoding the glycosyltransferase that add the lycotetraose moiety to tomatidine. The potato has a similar cluster in the syntenic region, however potato lacks orthologs to the *GAME18* and *GAME2* genes of tomato, which mediate the two final steps of tomatine biosynthesis (Cardenas et al., 2015). Also the genes *GAME4* and *GAME12*, involved in the first two dedicated steps towards glycoalkaloid synthesis, are physically clustered in tomato chromosome 12, and this cluster is conserved in potato (Cárdenas et al., 2015).

The regulation of α-tomatine metabolism involves the *GAME9* gene, a member of the APETALA2/Ethylene Response Factors family, also referred to as *JRE4* (Cárdenas et al., 2016; Thagun et al., 2016). More recently, additional genes involved in the (positive or negative) regulation of α-tomatine metabolism have been identified (Chen et al., 2019; Swinnen; et al., 2020; Wang et al., 2018; Zhao et al., 2018; see **Table 1**).

Table 1. Tomato genes shown to be involved in regulation of glyoalkaloid biosynthesis.

Gene name	Locus name	Role in regulation of α-tomatine metabolism	Target genes	References
GAME9/ JRE4	Solyc01g090340	positive regulator	*C5-SD, DWF5, GAME4, GAME7, GAME17*	Cárdenas et al. (2016); Nakayasu et al. (2018); Thagun et al. (2016) Yu et al. (2020)
MYC2	Solyc08g076930	positive regulator	*C5-SD, GAME4, GAME7* (requires GAME9)	Cárdenas et al. (2016); Swinnen et al. (2020)
MYC1	Solyc08g005050	positive regulator	*C5-SD*	Swinnen et al. (2020)
TAGL1	Solyc07g055920	negative regulator	n/a	Zhao et al. (2018)
TDR4/FUL1	Solyc06g069430	negative regulator	n/a	Zhao et al. (2019)
HY5	Solyc08g061130	positive regulator	*GAME1, GAME4, GAME17*	Wang et al. (2018)
PIF3	Solyc01g102300	negative regulator	*GAME1, GAME4, GAME17*	Wang et al. (2018)
MYB12	Solyc06g009710	positive regulator	n/a	Chen et al. (2019)

Toxicity mechanisms: membrane disruption or more than that?

Numerous studies have shown that α-tomatine is toxic to a spectrum of tomato pathogens and pests. Membrane disruption followed by cytoplasmic leakage and cell death was observed in cells exposed to α-tomatine (Arneson & Durbin, 1968; Campbell & Duffey, 1979; Hoagland, 2009; Osbourn, 1996a). The molecular basis for membranolytic action has been studied in depth. Membrane leakage caused by α-tomatine is dependent on the presence of sterols in the plasma membrane. A mutant of *Fusarium solani* which accumulated 20% less sterol in the membrane manifested lower sensitivity to α-tomatine (Défago & Kern, 1983). *Phytophthora* species do not synthesize sterols and hence were tolerant to α-tomatine, however, they gained sensitivity when grown in medium supplemented with free sterols (Steel & Drysdale, 1988). Old studies showed that α-tomatine can bind *in vitro* to different types of sterols such as cholesterol and ergosterol which are the major mammalian and fungal sterols, respectively, and to sitosterol and stigmasterol that are predominantly found in plant cells (Roddick, 1979). The membrane disrupting effect

caused by this interaction required the intact tetrasaccharide group of α-tomatine and the presence of sterol 3β-hydroxy groups (Nepal et al., 2019). By contrast, membranes containing sterols lacking 3β-hydroxy groups were insensitive to disruption by α-tomatine (Keukens et al., 1995; Roddick & Drysdale, 1984; Steel & Drysdale, 1988). Chemical hydrolysis products of α-tomatine lacking a single monosaccharide (β_1-tomatine, β_2-tomatine) or multiple sugars (γ-tomatine and the aglycon tomatidine) showed >95% reduction in their ability to disrupt membranes (Keukens et al., 1995).

Despite its disrupting activity on artificial membranes, infiltration of α-tomatine into the apoplast of tomato leaves did not cause visible damage (Ökmen et al., 2013). Considering the high concentration of α-tomatine in tomato tissue, tomato plants must be able to avoid self-intoxication. Indeed, tomato and potato leaves had a lower content of free sterols (~10%) and were more resistant to α-tomatine as they manifested less electrolyte leakage than plants containing higher proportions of free sterols such as tobacco and *Nicandra physalodes* (~50%) (Steel & Drysdale, 1988). The fact that tomato cells can withstand high concentrations of α-tomatine is likely associated with substitution at 3β-hydroxyl groups, thereby forming sterol conjugates which prevent binding with α-tomatine (Steel & Drysdale, 1988). In contrast to plants, fungi predominantly accumulate ergosterol, which occurs as free sterol and in multiple esterified forms (Hartmann, 1998; Weete et al, 2010). The ratio between these two forms varies among fungal species, but it is unknown whether this ratio affects the sensitivity to α-tomatine (Yuan et al., 2007).

Apart from the membranolytic action by sterol binding, Ito et al. (2007) reported that α-tomatine may induce programmed cell death (PCD) in the fungus *F. oxysporum*. Hallmarks of apoptosis such as DNA fragmentation, depolarization of the transmembrane potential of mitochondria and generation of reactive oxygen species (ROS) were detected in fungal cells treated with α-tomatine (Ito et al., 2007). The cell death induction by α-tomatine in *F. oxysporum* was markedly reduced by the application of a specific inhibitor of PCD (Ito et al., 2007), the only report thus far of PCD induction by α-tomatine in tomato pathogens. However, the PCD-inducing activity in fungi is not unexpected as α-tomatine was also reported to stimulate caspase-independent PCD in mouse colon cells and human leukemia cell lines (Chao et al., 2012; Kim et al., 2015). The induction of apoptosis in plant pathogens by plant defense molecules was reported in the interaction between *Arabidopsis* and the fungus *Botrytis cinerea* (Shlezinger et al., 2011). The toxic effect of α-tomatine seems to be based both on membranolytic activity and the activation of the PCD machinery, although the mechanism of PCD induction remains elusive. More efforts should be made to increase our understanding of the modes of action of α-tomatine.

How pathogens deal with α-tomatine: the role of tomatinase

One way of dealing with the toxic action of α-tomatine is the accumulation of low levels of sterols in membranes, as occurring in *Pythium* and *Phytophthora* species (Défago & Kern, 1983; Steel & Drysdale, 1988). Besides such passive tolerance, fungi can actively repair membrane damage inflicted by α-tomatine. Exposure of *Neurospora crassa* to α-tomatine triggered the recruitment of the membrane repair protein PEF1 to the lysing point at the membrane and deletion of *pef*1 increased the sensitivity of *N. crassa* to α-tomatine and other pore-forming drugs (Schumann et al., 2019). Furthermore, fungi can actively export exogenous toxic compounds through ATP-binding cassette (ABC) transporters. The roles of ABC transporters in the efflux of plant secondary metabolites and synthetic fungicides are well documented (Andrade et al., 2000; Kretschmer et al., 2009; Schoonbeek et al., 2001; Stefanato et al., 2009; Stergiopoulos & de Waard, 2002). In the insect herbivore *Helicoverpa armigera,* transcript levels for ABC transporters were induced when larvae were fed α-tomatine (Bretschneider et al., 2016), indicating that the adaption to α-tomatine in *H. armigera* might require ABC tranporters. However, a direct role of ABC transporters in the tolerance to α-tomatine in tomato pathogens and pests remains to be characterized.

Another active way of dealing with α-tomatine is to secrete enzymes that degrade α-tomatine to reduce its toxicity (Osbourn, 1996a; Sandrock & VanEtten, 1998). Hydrolysis of α-tomatine was first reported in the fungus *Septoria lycopersici* (Arneson, 1967). Since then, the identification and characterization of tomatinase activity was extended to more pathogens. Although enzymes catalyzing the hydrolysis of α-tomatine are collectively referred to as tomatinase, they differ in the glycosidic cleavage sites, catalytic mechanisms and the classification of corresponding genes (**Figure 2** and **Table 2**). The degradation process is categorized into three main actions based on the hydrolysis products: β_2-tomatine, β_1-tomatine and tomatidine as discussed below.

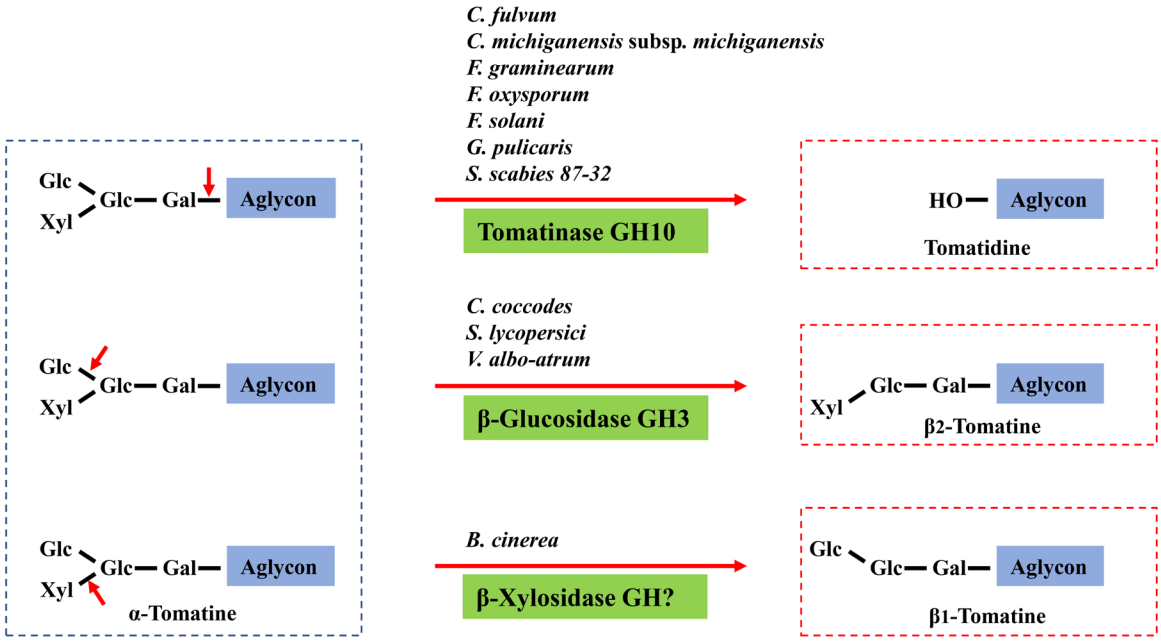

Figure 2. Different hydrolytic activities that detoxify α-tomatine. Chemical structures of α-tomatine are simplified. The glycosidic bonds that are cleaved are indicated by a red arrows. CAZyme activities that catalyze the reaction are indicated below the arrows, microorganisms that were shown to possess these activities are specified above the arrows.

Table 2. Microbial Glycosyl Hydrolases capable of degrading α-tomatine.

Pathogen	GH family	Accession (database)	Degradation product	Reference
Colletotrichum coccodes	n/a	n/a	β_2-tomatine	Sandrock & VanEtten (2001)
Septoria lycopersici	GH3	U35462 (NCBI)	β_2-tomatine	Sandrock et al. (1995)
Verticillium albo-atrum	n/a	n/a	β_2-tomatine	Pegg and Woodward (1986)
Botrytis cinerea	GH3, GH39, GH43?	n/a	β_1-tomatine	Quidde et al. (1998)
Cladosporium fulvum	GH10	188986 (JGI)	tomatidine	Ökmen et al. (2013)
Clavibacter michiganensis subsp. *michiganensis*	GH10	AAP57293 (NCBI)	tomatidine	Kaup et al. (2005)
Fusarium graminearum	GH10	EYB27127 (NCBI)	tomatidine	Carere et al. (2017)
Fusarium oxysporum f. sp. *lycopersici*	GH10	AJ012668 (NCBI)	tomatidine	Roldán-Arjona et al. (1999)
Fusarium solani	n/a	n/a	tomatidine	Lairini and Ruiz-Rubio (1998)
Gibberella pulicaris	n/a	n/a	tomatidine	Weltring et al. (1998)
Streptomyces scabies 87-22	GH10	CBG74701	tomatidine	Seipke & Loria (2008)
Alternaria alternata	n/a	n/a	unknown but not tomatidine	Oka et al. (2006)
Corynespora cassiicola	n/a	n/a	unknown but not tomatidine	Oka et al. (2006)

β_2-tomatine as the main product: generation of β_2-tomatine by cleaving off the terminal D-glucose has been reported in the fungi *S. lycopersici*, *Verticillium albo-atrum* and *Colletotrichum coccodes* (Arneson, 1967; Sandrock et al., 1995; Sandrock & vanEtten, 2001; Woodward & Pegg, 1986). The enzyme possesses β-glucosidase activity and is named as β_2-tomatinase. The cloning and sequencing of the *S. lycopersici* β_2-tomatinase gene indicated that it belongs to the Glycosyl Hydrolase (GH) family 3 of Carbohydrate Active enzymes (CAZY) (Lombard et al., 2013).

β_1-tomatine as the main product: besides the terminal D-glucose, the other terminal sugar moiety, D-xylose, can be the target of enzymatic hydrolysis. Removal of the terminal D-xylose and release of β_1-tomatine was reported in *B. cinerea* (Quidde et al., 1998). Genes encoding β_1-tomatinase have not been cloned so we cannot yet attribute the activity to a GH family, however, this enzyme must possess β-xylosidase activity and is likely from the GH39 or GH43 family of CAZYmes. Using the sequence of *S. lycopersici* β_2-tomatinase as a probe, Quidde et al. (1999) cloned a homolog (*sap1*) from *B. cinerea*. Characterization of a *B. cinerea sap1* knockout mutant revealed that this gene is not responsible for the β_1-tomatinase activity as the mutant can still hydrolyse α-tomatine (Quidde et al., 1999). The characterization of genes encoding β_1-tomatinase in *B. cinerea* and other fungi awaits their cloning.

Tomatidine as the main product: besides hydrolytic removal of single terminal sugar moieties, several microorganisms can convert α-tomatine to the aglycon tomatidine (**Figure 2** and **Table 2**): the fungi *Cladosporium fulvum* (Ökmen et al., 2013), *F. oxysporum* (Roldán-Arjona et al., 1999), *F. solani* (Lairini & Ruiz-Rubio, 1998)*, F. graminearum* (Carere et al., 2017) and *Gibberella pulicaris* (Weltring et al., 1998) as well as the bacterial pathogens *Clavibacter michiganensis* (Kaup et al., 2005) and *Streptomyces scabies* (Seipke & Loria, 2008). The generation of tomatidine is through the removal of the tetrasaccharide chain (lycotetraose). Unlike the β_2-tomatinase which belongs to the CAZY GH3 category, all tomatinase activities that detoxify α-tomatine through cleaving off the lycotetraose belong to the GH10 family (Table 2). At last, there is one example of an organism possessing distinct, functionally redundant enzymes capable of detoxifying α-tomatine. Removing the lycotetraose group was first considered to be the mode of action of α-tomatine degradation in *F. oxysporum* (Roldán-Arjona et al., 1999). However, a knockout mutant in the GH10 CAZyme gene remained able to degrade α-tomatine because it also possesses several GH3 enzymes that convert α-tomatine into β_2-tomatine instead of tomatidine. The β_2-tomatinase GH3 activity was not identified in the first place as it was masked by the presence of the GH10 tomatinase activity which cleaves off the entire lycotetraose branch (Pareja-Jaime et al., 2008).

Several aspects of the regulation of expression of tomatinase genes have been described. First of all, the tomatinase activity and expression of tomatinase genes can often be induced by α-tomatine (Ökmen et

al., 2013; Quidde et al., 1998; Roldán-Arjona et al., 1999). Secondly, induction seemed to be specific to α-tomatine treatment as there was no induction by other saponins such as chaconine or solanine in *B. cinerea* (Quidde et al., 1998). Finally, the effect of carbon catabolite repression differed between fungi. β_1-tomatinase from *B. cinerea* was not subject to catabolite repression (Quidde et al., 1998), however, expression of the *F. oxysporum* GH10 tomatinase gene was repressed when glucose is present (Roldán-Arjona et al., 1999).

Degradation of α-tomatine is more than detoxification

Pathogens achieve detoxification of α-tomatine by enzymatically converting it to less toxic products. In addition to detoxification, tomatinase activity may have additional biological repercussions. A GH3 tomatinase-deficient mutant of *S. lycopersici* caused more intense plant cell death than the wild type in early stages of infection and induced enhanced expression of defense-related genes on tomato leaves (Martin-Hernandez et al., 2000). In a different study, inoculation on *Nicotiana benthamiana* of the *S. lycopersici* GH3 tomatinase-deficient mutant, but not the wild type, elicited intense cell death in mesophyll tissue resembling a hypersensitive response and the infection was fully contained within 2 days post-inoculation (Bouarab et al., 2002). These observations indicated that GH3 tomatinase not only detoxifies α-tomatine, but also mediates the suppression of plant defense responses. Further experiments showed that pre-infiltration of β_2-tomatine in *N. benthamiana* leaves enabled the *S. lycopersicii* GH3 tomatinase mutant to cause expanding lesions and also compromised plant resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tabaci*. By contrast, infiltration of α-tomatine did not have such effects (Bouarab et al., 2002). Moreover, silencing of the *N. benthamiana* SGT1 gene, required for disease resistance in plants, restored the pathogenicity of *S. lycopersici* GH3 tomatinase-deficient mutant (Austin et al., 2002; Bouarab et al., 2002; Peart et al., 2002). These observations suggest that the capacity of tomatinase to suppress plant defense depends on the breakdown product(s) generated by tomatinase rather than the protein itself. A dual function of tomatinase was also reported in *F. oxysporum* which converts α-tomatine by a GH10 hydrolase to the aglycon tomatidine (Roldán-Arjona et al., 1999). The addition of either tomatidine or lycotetraose to suspension-cultured tomato cells can suppress the oxidative burst as well as hypersensitive cell death triggered by fungal elicitor (Ito et al., 2004). The effect of tomatidine and lycotetraose on the production of ROS was studied in more detail. *In vitro* assays revealed that tomatidine could scavenge superoxide anions as effectively as ascorbic acid, whereas lycotetraose did not possess antioxidant activity. These observations suggest that the suppression of an oxidative burst by degradation products of α-tomatine is based on different mechanisms: tomatidine can

directly scavenge ROS while lycotetraose might block the generation of ROS through an as yet unknown mechanism. Furthermore, treatment of tomato plant with tomatidine or lycotetraose promoted the colonization of hypocotyls by a non-pathogenic *F. oxysporum* strain lacking tomatinase activity (Ito et al., 2004). Besides modulating plant defense responses, tomatidine was reported to exhibit phytotoxic effects. Transgenic tomato plants in which the *GAME1* gene (**Figure 1**) was silenced, accumulated excessive levels of tomatidine and exhibited severe developmental defects (Itkin et al., 2011). This observation was substantiated by the cell death-inducing effect on tomato leaves of exogenously applied tomatidine (**Figure 3**; Ökmen et al., 2013). Based on these studies it is apparent that the hydrolysis of α-tomatine during pathogen infection is not merely reducing its toxicity but also affecting the physiology and defense responses of the plant through α-tomatine breakdown products. In some situations, the latter role appeared to be important for virulence of tomato pathogens (Bouarab et al., 2002; Ito et al., 2004).

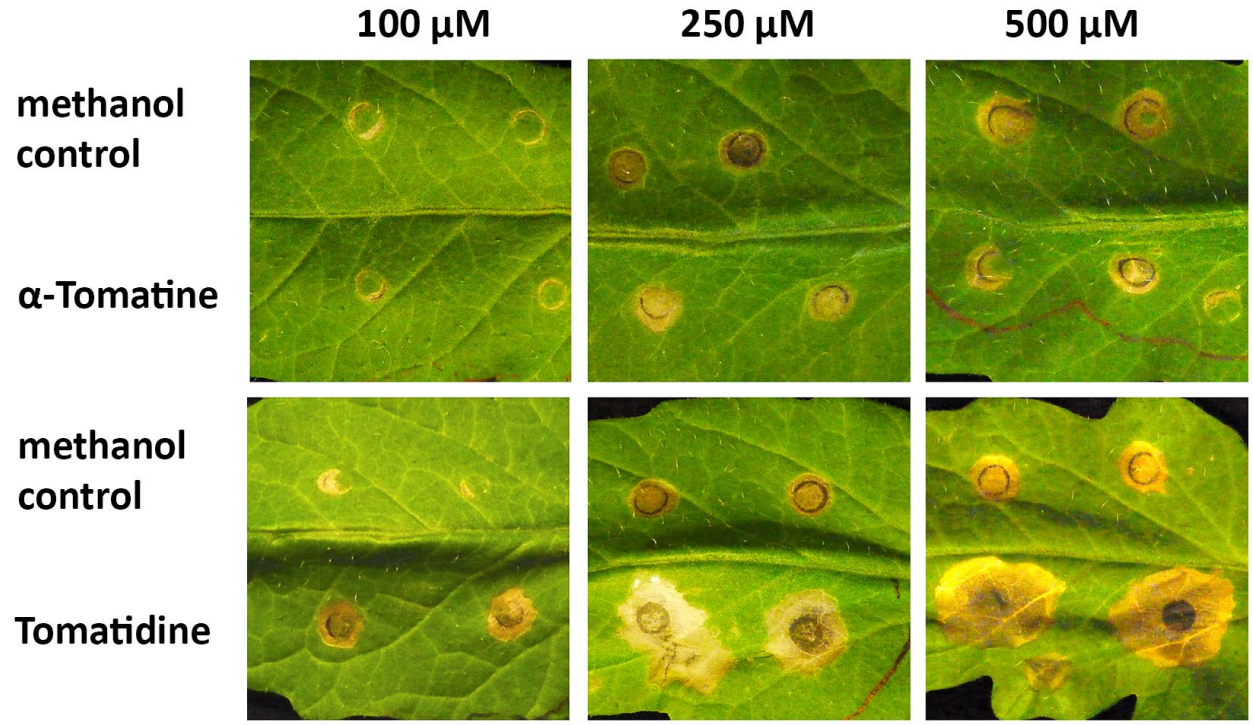

Figure 3. Phytotoxic effects of α-tomatine and tomatidine on tomato leaves. Taken from Ökmen et al. (2013) with permission.

Subcellular localization: arsenal or battlefield?

Like other saponins, α-tomatine is thought to be localized within tomato cells and to be released upon cell damage resulting from pathogen invasion (Dow & Callow, 1978) or pest feeding. The subcellular localization can define the spatial and temporal contribution of α-tomatine to the inhibition of pathogen infection. Theoretically, if a tomato pathogen can avoid the release of α-tomatine from the host cells, it would circumvent the inhibition. Studies on the biotrophic tomato pathogen *C. fulvum* have shed light on the importance of the distribution of α-tomatine because this fungus exclusively colonizes the apoplast and causes limited damage to host cells until the final stage of infection (Stergiopoulos & de Wit, 2009). Initially, it was proposed that *C. fulvum* was less likely to encounter inhibitory concentrations of α-tomatine during infection if the glycoalkaloid predominantly localizes intracellularly. Based on this assumption, it was hypothesized that tomatinase activity might not be important for full virulence of *C. fulvum* despite high sensitivity of the fungus to α-tomatine (Dow & Callow, 1978; Melton et al., 1998; Kohmoto & Yoder, 1998). In order to test whether tomatinase activity contributes to virulence, Melton et al. (1998) expressed a GH3 tomatinase gene from *S. lycopersici* in *C. fulvum* as this was the only characterized tomatinase gene at that time. Expression of the heterologous tomatinase resulted in enhanced virulence of *C. fulvum* (as assessed by increased sporulation) and provided evidence for a positive role of α-tomatine degradation to *C. fulvum* infection (Melton et al., 1998). A later study described the identification of the *C. fulvum* endogenous GH10 tomatinase gene *CfTom1* and further substantiated the role of tomatinase activity. A knockout mutant in the *CfTom1* gene displayed increased sensitivity against α-tomatine and reduced virulence on tomato (Ökmen et al., 2013). This study also detected the presence of α-tomatine in apoplastic fluid at 0.02mM, which is low as compared to the levels of ~ 1mM in total leaf extract (Ökmen et al., 2013). In light of these studies, there is no doubt that α-tomatine predominantly accumulates inside plant cells, however, the amounts of α-tomatine in intercellular spaces might be sufficient to exert some inhibition to invading microbes. It is unknown whether the apoplastic localization of α-tomatine involves active secretion or merely results from the leakage from cells. The impact of the intercellular distribution of α-tomatine in the defense against pathogens that employ different infection strategies such as necrotrophic and hemibiotrophic pathogens, remains unclear.

Effect of pH on tolerance to α-tomatine

α-Tomatine is more toxic at higher pH (Arneson & Durbin, 1968; Dow & Callow 1978). At pH 3.0, almost 300 times higher α-tomatine concentrations were required to achieve the same inhibitory effect on fungi as at pH 8.0 (Arneson & Durbin, 1968). This effect might be partially caused by increased protonation of

α-tomatine in acidic conditions, as only unprotonated α-tomatine can bind to cholesterol *in vitro*, whereas the protonated form could not bind cholesterol (Arneson & Durbin, 1968). Besides influencing the toxicity of α-tomatine, ambient pH may also affect the expression of tomatinase genes. The *C. fulvum* GH10 tomatinase gene *Cftom1* was barely expressed in liquid medium containing α-tomatine at pH 4.0, whereas abundant transcript levels were detected at pH 7 (Ökmen et al., 2013). This observation explained why a previous study could not detect α-tomatine degradation as the medium used to grow the mycelium was adjusted to pH 4.5 (Melton et al., 1998). Studies showing the impact of pH on *in vitro* assays raise questions about the role of ambient pH at infection sites in tomato-pathogen interactions, and highlight the possible impact of ambient pH manipulation by microbes during infection, such as the host tissue acidification reported for *B. cinerea* (Müller et al., 2018). Although the effect of pH manipulation might not occur with the specific purpose to decrease sensitivity to α-tomatine, it likely affects its toxicity and thereby could have an impact on the outcome of tomato-microbe interactions.

Typical phytoanticipin or more than that?

The term phytoanticipin was first proposed and defined by VanEtten et al. (1994). Phytoanticipins are low molecular weight metabolites with antibiotic properties, that are either preformed or generated from accumulated precursors when plants are challenged by pathogens. Phytoanticipins differ from the phytoalexins, which are induced upon pathogen infection. α-tomatine has long been considered as a potent phytoanticipin because of its high accumulation in healthy tomato tissues and its toxicity against different pathogens (Osbourn, 1996b; Piasecka et al., 2015). A recent study on resistance to early blight (*Alternaria solani*) described a difference in metabolic profiles between a resistant wild tomato (*Solanum arcanum*) and the susceptible cultivated tomato (*S. lycopersicum*). This study indicated that α-tomatine can also serve as a phytoalexin in certain conditions (Shinde et al., 2017). A pronounced increase of α-tomatine content was detected in *S. arcanum* after *A. solani* infection, to levels 10 times higher than before infection. By contrast, the susceptible cultivated tomato had more severe symptoms, and its α-tomatine level increased by only 2.5 fold. Counter-intuitively, the expression of α-tomatine biosynthetic genes *GAME1*, *GAME17* and *GAME18,* as well as the regulator gene *GAME9,* were much higher in susceptible cultivated tomato despite the lower increase of α-tomatine levels, as compared to the wild tomato upon infection. In contrast, *GAME2* which encodes the enzyme that performs the last step of α-tomatine synthesis was expressed at much higher levels in resistant wild tomato, highlighting an important (rate-limiting) role of *GAME2* expression in α-tomatine stimulation in response to *A. solani* invasion. To date, this is the only report showing that α-tomatine biosynthesis can be elicited by the challenge of microbes.

The balance between α-tomatine synthesis and degradation defines the outcome on the battlefield

Although being referred to as a defense compound in many studies because of its high accumulation in tomato tissue and its toxicity against many pathogens, direct evidence of the contribution of α-tomatine to plant immunity is lacking. The importance of α-tomatine in basal defense is indirectly implied from various studies on tomato pathogens. Firstly, tomato pathogens tend to be more resistant to α-tomatine than organisms that are non-pathogenic on tomato (Arneson & Durbin, 1968; Steel & Drysdale, 1988). For instance, mycelia of the fungal tomato pathogens *B. cinerea*, *V. albo-atrum* and *F. solani* exhibited less electrolyte leakage than non-tomato pathogens such as *A. tenuis*, *Ascochyta pisi* and *F. graminearum* when incubated with α-tomatine (Steel & Drysdale, 1988). A comprehensive study among 23 fungal strains revealed a strong correlation between the tolerance to α-tomatine, the ability to degrade α-tomatine and pathogenicity on tomato (Sandrock & vanEtten, 1998). A similar phenomenon was observed in pea pathogens: among 50 plant pathogenic microbes, only the taxa that were able to metabolize the pea phytoalexin pisatin could infect pea, and all isolates that were non-pathogenic on pea were unable to detoxify pisatin (Delserone et al., 1999). Moreover, the pea pathogen *Nectria haematococca* can infect mature tomato fruit (low in α-tomatine) but not green fruit, which accumulates high concentrations of α-tomatine. Whereas expression of the *S. lycopersici* GH3 tomatinase gene in *N. haematococca* conferred the ability to colonize green tomato fruit (Sandrock & vanEtten, 2001). These observations implicate that degrading α-tomatine is essential to achieve successful infection on tomato or determining the host range. A similar concept was described for the oat pathogen *Gaeumannomyces graminis* var. *tritici*, in which mutants that were unable to degrade the oat saponin avenacin A-1 lost their ability to infect oat (Osbourn et al., 1995).

However, the mutagenesis of genes encoding tomatinase in several microbes thus far did not support the role of tomatinase as an essential determinant in pathogenicity on tomato but rather acts as a potential virulence factor. For example, *C. fulvum* GH10 tomatinase-deficient mutants remained pathogenic on tomato despite accumulating less fungal biomass, whereas the heterologous overexpression of *S. lycopersici* GH3 tomatinase in *C. fulvum* enhanced fungal sporulation during tomato infection (Melton et al., 1998). Also in *F. oxysporum*, GH10 tomatinase-deficient mutant caused delayed disease development as compared to the wild type (Pareja-Jaime et al., 2008). Moreover, the natural field isolate M3a of *B. cinerea* (from grape) was deficient in α-tomatine degrading activity and accordingly was less virulent on tomato leaves, as compared to the α-tomatine degrading strain B05.10. When infecting plant tissues lacking α-tomatine such as bean leaves, similar lesion sizes were observed for M3a and B05.10 (Quidde et al., 1998). In addition, the infection on tomato was unaffected when tomatinase was disrupted in *S.*

lycopersici (Martin-Hernandez et al., 2000), or in the bacterium *S. scabies* (Seipke & Loria, 2008). In these cases, it suggested that these organisms possess additional mechanisms that confer tolerance to α-tomatine. These observations are indicative of the importance of tomatinase in tomato-microbe interactions and consequently highlight the potential role of α-tomatine in tomato basal defense.

Moreover, the contribution of "tomatinase" to plant infection might not necessarily be (exclusively) related to α-tomatine degradation. As described above, tomatinases are glycosyl hydrolases of distinct CAZyme families, which might also act on substrates other than α-tomatine and thereby play a different role in the infection. For instance, the virulence of GH3 tomatinase-deficient mutants of *S. lycopersici* was not reduced on tomato leaves, however, they failed to infect *N. benthamiana* leaves which do not accumulate α-tomatine (Bouarab et al., 2002). Similarly, a glycosyl hydrolase from *F. graminearum* possessing hydrolytic activity an α-tomatine acted as a virulence factor on wheat (Carere et al., 2017).

In a recent study, tomato leaves overexpressing a gene encoding tomato strictosidine synthase (STR-2) accumulated more α-tomatine and exhibited enhanced resistance against *B. cinerea* and *P. infestans* (Chen et al., 2019). Taken together, there are strong indications that α-tomatine participates in the basal defenses against pathogens. However, whether increasing α-tomatine levels may increase resistance to presently notorious pathogens and the absence of α-tomatine will render tomato plants more susceptible to organisms not normally infecting tomato remains to be studied. Such studies would benefit from using α-tomatine-deficient transgenic tomato (using CRISPR) as well as lines accumulating a higher level of α-tomatine to provide direct evidence of the role of α-tomatine in plant immunity.

Conclusion and perspectives

The data discussed above provide circumstantial evidence that α-tomatine is a specialized metabolite that confers important levels of protection from herbivory and pathogen invasion. The final proof of its important function in plant defense remains to be provided by knocking out its biosynthesis or increasing its levels by selective overexpression of *GAME* genes and testing the impact of altered α-tomatine levels on the susceptibility to herbivores or pathogens. The mode of action on plant, microbial and insect membrane and the mechanisms by which α-tomatine induces PCD in plants need to be resolved.

Enzymatic degradation of α-tomatine by three distinct types of microbial secreted CAZymes (GH3, GH10 or GH43) that, respectively, remove the terminal glucose or xylose moieties, or the entire lycotetraose group provides a beautiful example of independent, convergent evolution in several pathogenic bacteria and fungi towards the detoxification of a potent antimicrobial compound. These genes probably evolved from ancestral GH genes with the appropriate catalytic site, towards specialization on a substrate that was

a major obstacle for pathogen development and reproduction in a toxic environment. For detoxification of other phytoanticipins, such as avenacin and pisatin, there is generally just a single enzymatic activity reported that can inactivate these compounds. The finding of three separate detoxification activities for the same antimicrobial compound is remarkable.

It is noteworthy that enzymatic degradation products of α-tomatine such as β_2-tomatine, tomatidine and lycotetraose can modulate the immune response of a plant, suggesting that the removal of sugar moieties benefits a pathogenic microbe in two ways, by reducing membrane permeating activity of α-tomatine ánd lowering the plant defense machinery. The impact of antimicrobial plant metabolites on the plant immune response through different mechanisms (other than being toxic to microbes) deserves further attention. The observation that sterol glycosylation in tomato confers tolerance to the toxicity of α-tomatine raises the question whether microbes, and especially tomato pathogens, could protect themselves from membrane damage by glycosylating their sterols, either constitutively or in the presence of α-tomatine.

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Chapter 5

Multiple mechanisms for tolerance to α-tomatine in *Botrytis cinerea* contribute to pathogenicity on tomato

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Abstract

α-Tomatine, a steroidal glycoalkaloid saponin, is abundant in vegetative tissues of tomato and has antibiotic activities. Secretion of glycosyl hydrolases (GH) to enzymatically degrade α-tomatine into less toxic products is the only mechanism reported in tomato pathogens so far. In this study, we identified α-tomatine-responsive genes in isolate B05.10 by RNA-sequencing. We observed strong induction of genes encoding a GH from the GH43 family, a glycosyl transferases (GT) from the GT28 family as well as membrane proteins including one ABC transporter and multiple RTA1-like proteins. In addition, the genome sequencing of *B. cinerea* grape isolate M3a, which is unable to degrade α-tomatine, revealed a missing locus compared with B05.10 comprising the GH43 and GT28a due to the insertion of a transposable element. Recombinant GH43 protein exhibited *in vitro* tomatinase activity through hydrolysis of the terminal xyloside of α-tomatine. The B05.10 knockout (KO) mutant of *GH43* lost tomatinase activity, became more sensitive to α-tomatine and less virulent on tomato and therefore GH43 is designated as BcTom1. Moreover, the overexpression (OE) of either the *GH43/BcTom1* or the other two types of tomatinase genes from *Septoria lycopersici* (GH3) and *Cladosporium fulvum* (GH10) separately in M3a conferred tomatinase activities, and restored the virulence on tomato. The deletion of the *GT28a* in B05.10 reduced its virulence on tomato but did not impact α-tomatine sensitivity. However, OE of *GT28a* in M3a led to elevated resistance to α-tomatine and increased virulence on tomato. Upon α-tomatine treatment, the GT28a-GFP fusion protein was recruited from cytosol to the membranes of hyphal tips (abundant in ergosterol) indicating the importance of membrane modification in resistance to α-tomatine and virulence on tomato.

Introduction

Plants can produce a wide range of secondary metabolites. Some of these metabolites not only exhibit *in vitro* antimicrobial activities with diverse modes of action for preventing growth of microbes, but they have also been demonstrated to play an important defensive role in plant innate immunity (Piasecka et al., 2015). VanEtten (1994) grouped antimicrobial compounds in plants in two categories. The constitutive antimicrobial compounds that are preformed prior to biotic stresses were defined as phytoanticipins, whereas the term phytoalexins is used for antimicrobial metabolites of which the biosynthesis is induced when plants are under pathogen attack.

Saponins are a class of secondary metabolites comprising glycosylated triterpenoids, steroids or steroidal alkaloids. They display "soapy" behavior due to their amphiphilic characteristics, with a lipophilic aglycon tail and a hydrophilic oligosaccharide head group. Saponins are widely distributed in many plant species (Osbourn, 1996; Piasecka et al., 2015) and exhibit antibiotic activities against a wide range of plant pathogens and pests including fungi, bacteria, oomycetes, nematodes and herbivorous insects (Akinpelu et al., 2014; Augustin et al., 2012; D'Addabbo et al., 2011; Hussain et al., 2019; Osbourn, 1996; Potter and Kimmerer, 1989). As defense compounds, saponins are suggested to constitute an important layer of basal defense in plants (Hussain et al., 2019; Piasecka et al., 2015). Saponin-deficient (*sad*) mutants of the wild oat species *Avena strigosa* were significantly compromised in resistance against a variety of fungal pathogens (Papadopoulou et al., 1999). α-Tomatine, a steroidal glycoalkaloid (SGA) compound consisting of the steroidal aglycon tomatidine and a tetrasaccharide sugar chain (β-lycotetraose), is the most-studied saponin. It is present in tomato vegetative tissues and green fruit and its fresh weight concentration can exceed 1 mM (You and van Kan, 2021). Since the first report of its antifungal effect on *Fusarium oxysporum* f. sp. *lycopersici* (Roddick, 1974), numerous studies have demonstrated its broad antibiotic activity not only against various fungi but also include oomycetes, bacteria, viruses and insects (Bailly, 2021; Duffey and Stout, 1996; Hoagland, 2009; Sparg et al., 2004; Thorne et al., 1985; You and van Kan, 2021). The mode of action of antifungal activity of α-tomatine has been well-investigated and is proposed to be associated with membrane disruption following the complexing of α-tomatine with fungal sterols (Sandrock and VanEtten, 1998). In addition, α-tomatine was reported to induce programmed cell death in *Fusarium oxysporum* (Ito et al., 2007).

Enzymatic degradation of saponins via the hydrolysis of glycosides that converts them into less toxic breakdown products is the main detoxification strategy reported in phytopathogenic fungi. The removal of terminal glucosides from the oat saponin avenacin A-1 has been demonstrated in *Gaeumannomyces*

graminis var. *avenae* and is essential for its pathogenicity on oat but not on wheat which does not accumulate avenacin A-1 (Bowyer et al., 1995). Deglycosylation of α-tomatine has been reported in several tomato pathogens and is mediated by tomatinases from different glycosyl hydrolase (GH) families with distinct modes of action (You and van Kan, 2021). The hydrolysis of the intact tetrasaccharide and the yield of the aglycon tomatidine is catalyzed by GH10 family tomatinases in fungal pathogens including *Cladosporium fulvum*, *F. oxysporum* f. sp. *lycopersici*, *F. graminearum* and *F. solani* (Carere et al., 2017; Lairini and Ruiz-Rubio, 1998; Ökmen et al., 2013). Secondly, the terminal glucose can be cleaved off and cause the release of β_2-tomatine by tomatinases belonging to the GH3 family as reported in *Septoria lycopersici*, *Verticillium albo-atrum* and *Colletotrichum coccodes* (Woodward and Pegg, 1986; Sandrock and VanEtten, 2001; Sandrock et al., 1995). Thirdly, the generation of β_1-tomatine resulting from the removal of the terminal xyloside is uniquely reported for *Botrytis cinerea* (Quidde et al., 1998) though the β-xylosidase responsible for this tomatinase activity remains unknown. The significance of saponin degradation in virulence has been investigated in several pathogens. Avenacinase mutants of *G. graminis* var. *avenae* exhibited increased *in vitro* sensitivity to avenacin A-1 and lost their ability to infect oat but retained pathogenicity on wheat (Bowyer et al., 1995). The GH10 tomatinases in *C. fulvum* and *F. oxysporum* were not essential for pathogenicity but rather contributed to full virulence on tomato, as manifested by the less severe symptoms caused by the tomatinase-deficient mutants (Ökmen et al., 2013; Pareja-Jaime et al., 2008). In addition, the contribution of saponin-degrading enzymes to plant infection is not only by their role in detoxification but is also related to the suppression of plant defense responses mediated by the breakdown products (Bouarab et al., 2002; Ito et al., 2004).

It is important to note that the knowledge on fungal tolerance mechanisms to saponins is restricted to enzymatic degradation. Other non-hydrolytic strategies could exist and are potentially important for resistance to saponins and virulence on saponin-containing plants. For instance, the *S. lycopersici* tomatinase mutants were more sensitive to α-tomatine but their growth was not fully inhibited even at 1 mM, and the virulence on tomato was not reduced (Martin-Hernandez et al., 2000). Given the fact that saponins such as α-tomatine can disrupt fungal membrane integrity via binding to 3β-hydroxy sterols (free sterols), modification or loss of the targets (sterols) might lead to increased tolerance to saponins. This can be inferred from the relatively high tolerance to α-tomatine in *Phytophthora* species, which lack the biosynthetic capacity to synthesize sterols (Steel and Drysdale, 1988). Besides, it has also been implicated that tomato and potato cells can withstand high levels of α-tomatine and solanine, respectively, thanks to the presence of a high proportion of glycosylated sterols in their membranes (Walker et al., 2008; Steel and Drysdale, 1988). Furthermore, it has been reported that the *Neurospora crassa* PEF1 protein, involved

in membrane repair, can be recruited to fungal membranes upon α-tomatine treatment and is important for tolerance to α-tomatine (Schumann et al., 2019). Lastly, the involvement of plasma membrane efflux pumps in coping with saponins also requires investigation.

Botrytis cinerea, the causal agent of grey mould disease, is a broad host range fungal pathogen affecting the global food production (Williamson et al., 2007). As mentioned, it produces a unique type of tomatinase activity (Quidde et al., 1998) but the gene encoding the tomatinase remains to be identified. The availability of *B. cinerea* genome information combined with a recently established CRISPR/Cas9 mutagenesis system render this fungus an ideal model to study the tolerance mechanisms to saponins and their contribution to virulence (Leisen et al., 2020; van Kan et al., 2017). In this study, we investigated the α-tomatine-inducible gene expression in *B. cinerea* by RNA-sequencing (RNA-seq) analysis. Among the genes with up-regulated transcript levels upon α-tomatine treatment, we identified several genes with potential roles in conferring saponin tolerance. The functional analysis of these genes revealed that *B. cinerea* uses multiple mechanisms for tolerance to α-tomatine, each of which makes a quantitative contribution to the virulence of *B. cinerea* on tomato.

Results

α-Tomatine induces membrane disruption predominantly at the ergosterol-enriched domain

Because α-tomatine is known to cause membrane disruption through complexing with sterols, we first examined the distribution of sterols in the membrane of *B. cinerea* by filipin staining (**Figure 1**) and studied the pore-forming activity of α-tomatine on *B. cinerea*. B05.10 transformants were generated that overexpress *PEF1-GFP*, a protein that can be mobilized to damaged sites at the membrane as a response to the Ca^{2+} influx and thereby acts as a marker for membrane damage. The PEF1-GFP signal in fungal hyphae before α-tomatine treatment is mainly cytoplasmic (**Figure 2)**. After addition of α-tomatine, punctate GFP signal was observed at the ergosterol-enriched sites (filipin staining) of hyphae (**Figure 2**).

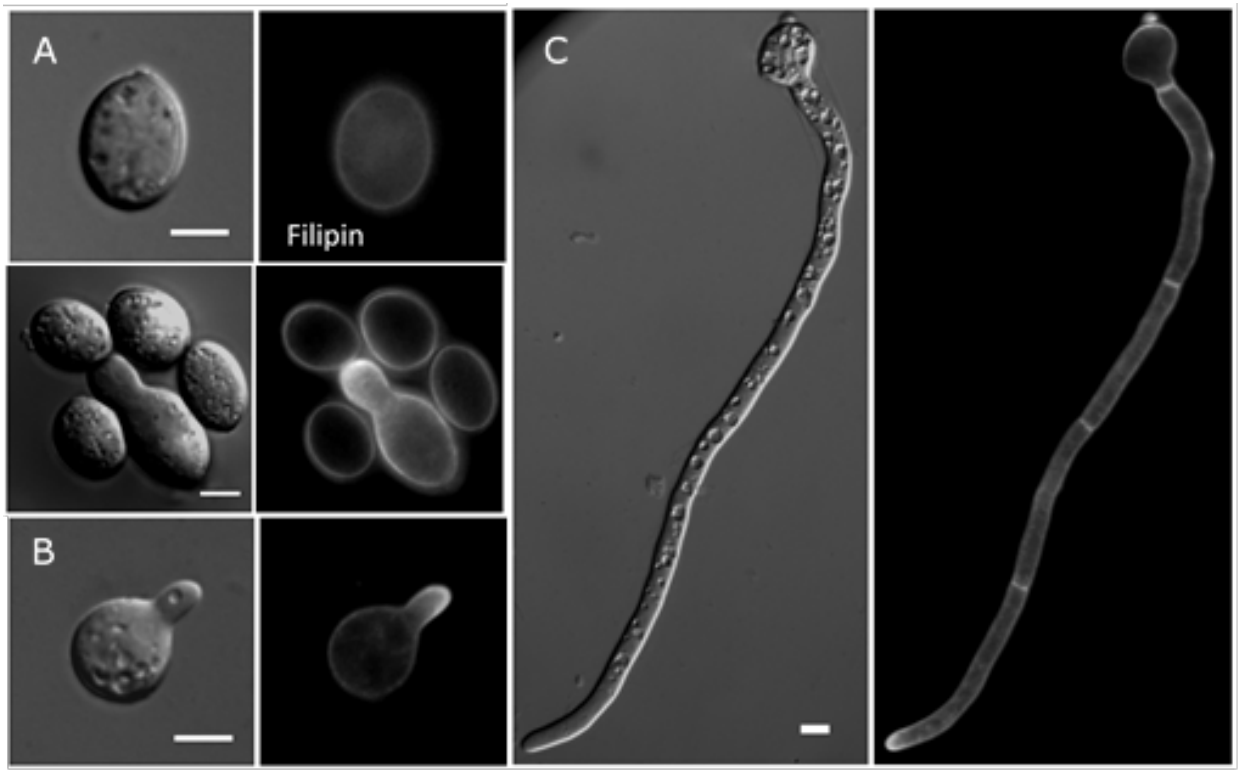


Figure 1. Filipin staining of *B. cinerea* spores (A), germlings (B) and hyphae (C). Accumulation of ergosterols is indicated by the fluorescence. The scale bar indicates a distance of 5 µm (A and B) or 2.5 µm (C)

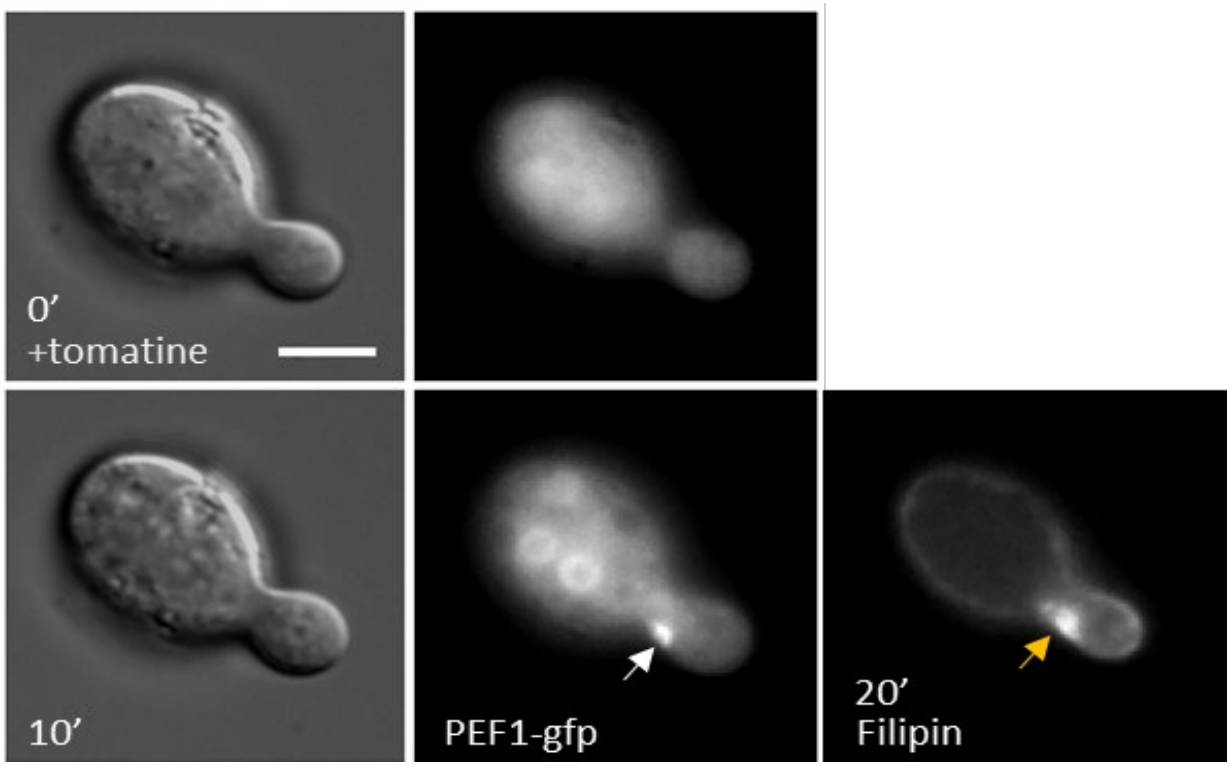


Figure 2. PEF1-GFP signal was observed at 10 mins after transformant treated with 190 µM α-tomatine. Then the germling was stained with filipin after 20 mins to indicate the presence of membrane ergosterol. Signals are indicated by white arrow (GFP) or yellow arrow (filipin staining). The scale bar indicates 5 µm.

Botrytis cinerea isolate B05.10 converts α-tomatine into β_1-tomatine, whereas isolate M3a lacks tomatinase activity

Previous studies by Quidde et al. (1998) identified a *B. cinerea* isolate, named M3a, which was unable to hydrolyze α-tomatine which is in contrast to many other isolates including B05.10. α-Tomatine was added to liquid cultures of B05.10 and M3a and aliquots of the culture fluids taken at different time points were analyzed for the presence of glycoalkaloids. After 9 h incubation with α-tomatine, the supernatant of the B05.10 culture contained β_1-tomatine as the only α-tomatine breakdown product indicating that tomatinase activity is conferred by a β-xylosidase. No hydrolytic conversion product of α-tomatine was identified in the supernatant of M3a liquid culture (**Figure 3)**.

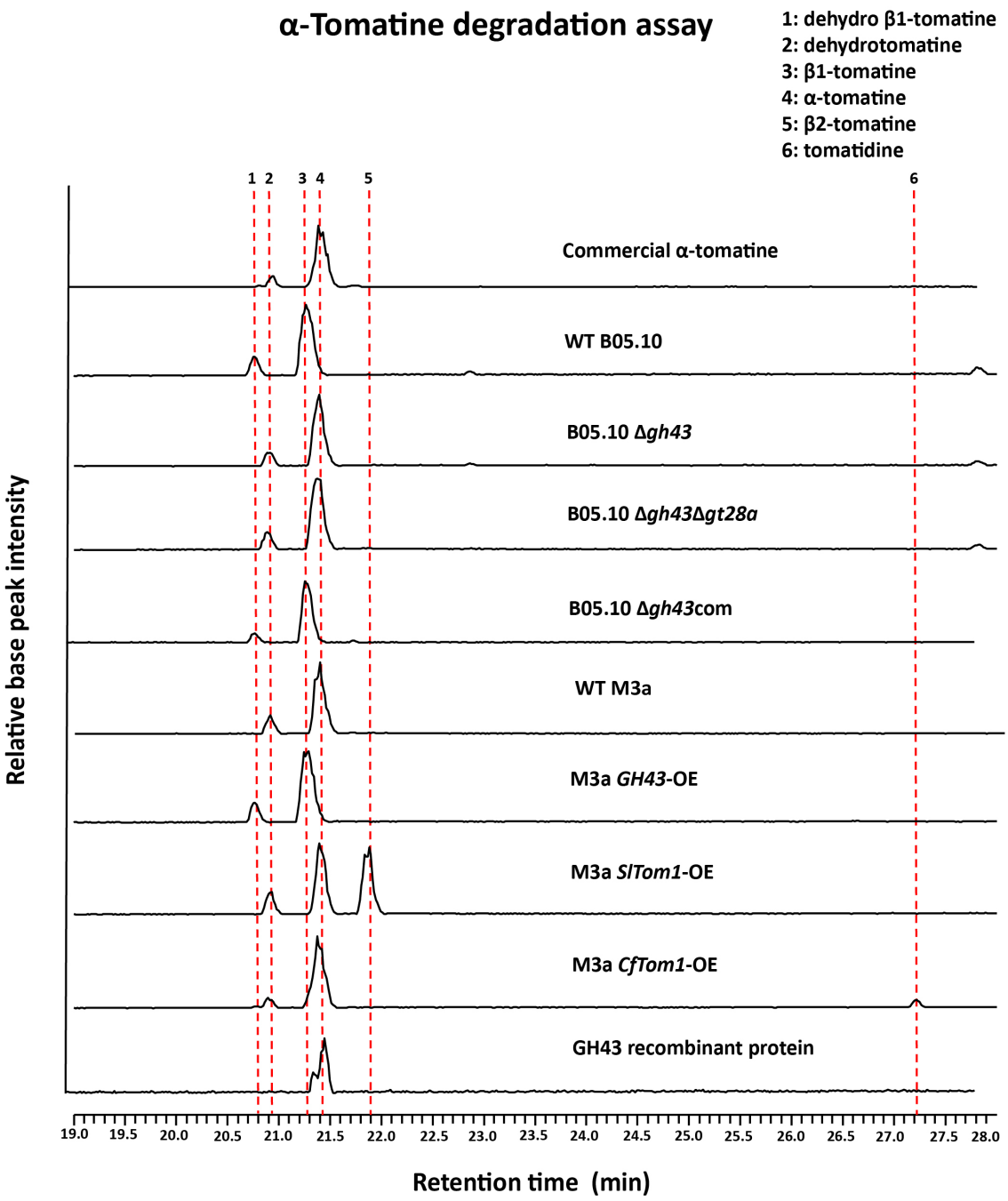


Figure 3. α-Tomatine degradation assay by LC-MS. *B. cinerea* overnight (ON) liquid culture were incubated with 200 µM α-tomatine for 9 hours (h) and the degradation products in the supernatant were detected by LC-MS.

M3a displays increased sensitivity to α-tomatine and compromised virulence on tomato

The sensitivity to α-tomatine was compared between B05.10 and M3a on agar plates containing different concentrations of α-tomatine. M3a was more sensitive to α-tomatine and digitonin than B05.10 (**Figure 4C**). Infection assays were carried out to investigate the role of tomatinase in pathogenicity. Virulence of

M3a on tomato leaves was severely compromised (**Figure 4A**). Under conditions where B05.10 infection displayed expanding lesions, M3a infection was mostly confined to pinpoint necrotic spots and could not cause expanding lesions on tomato leaves. M3a was able to cause expanding lesions on *Nicotiana benthamiana* leaves, although it exhibited smaller lesions than B05.10 (**Figure 4B**).

Genome comparison of M3a with B05.10 revealed diversity potentially involved in α-tomatine tolerance

The genome sequence of the tomatinase-producing isolate B05.10 serves as the reference genome for the species *B. cinerea* (van Kan et al., 2017). We sequenced the genome of isolate M3a using a combination of Illumina and Nanopore technology, and examined the assembled M3a genome for polymorphisms with isolate B05.10 (**Figure 5A**). Specifically, the assembly was examined for the absence of genes in M3a, or for polymorphisms that would render a B05.10 gene dysfunctional. The most obvious and striking difference that was observed was the absence in M3a of a region of 12 kb from B05.10 Chromosome (Chr) 8, which contains two genes: *GH43* (Bcin08g00060) and *GT28a* (Bcin08g00070), encoding a GH43 glycosyl hydrolase and a GT28 glycosyltransferase, respectively (**Figure 5B**). Besides, An indel was identified in the coding region of the *BcatrT* only in B05.10 which could cause the formation of a premature stop codon in the first ATPases Associated with diverse cellular Activities (AAA) domain (**Figure 5C**)

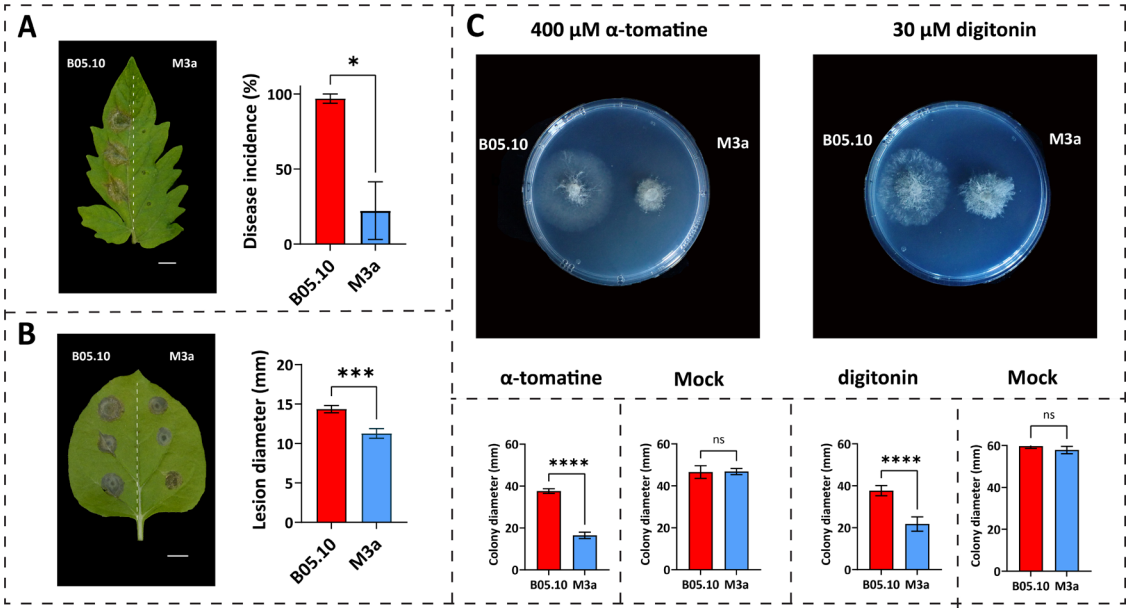


Figure 4. Characterization of virulence and sensitivity against plant toxic compounds in M3a as compared with B05.10. Infection of B05.10 and M3a on tomato (A) or *N. benthamiana* (B). Lesion diameters and disease incidences were based on three independent inoculation assays; Sensitivity test of *B. cinerea* B05.10 and M3a against digitonin or α-tomatine. Fungal radial growth was from 5 mm mycelial agar plugs inoculated on GB5-10 mM sucrose- 10 mM phosphate plates containing 30 µM digitonin (C) and 400 µM α-tomatine (D). Colony diameters were measured after 3 days. Error bars are standard error of mean (SEM) of all the biological replates. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; *** p-value <0.005; **** p-value <0.0001). ns indicates no significant difference.

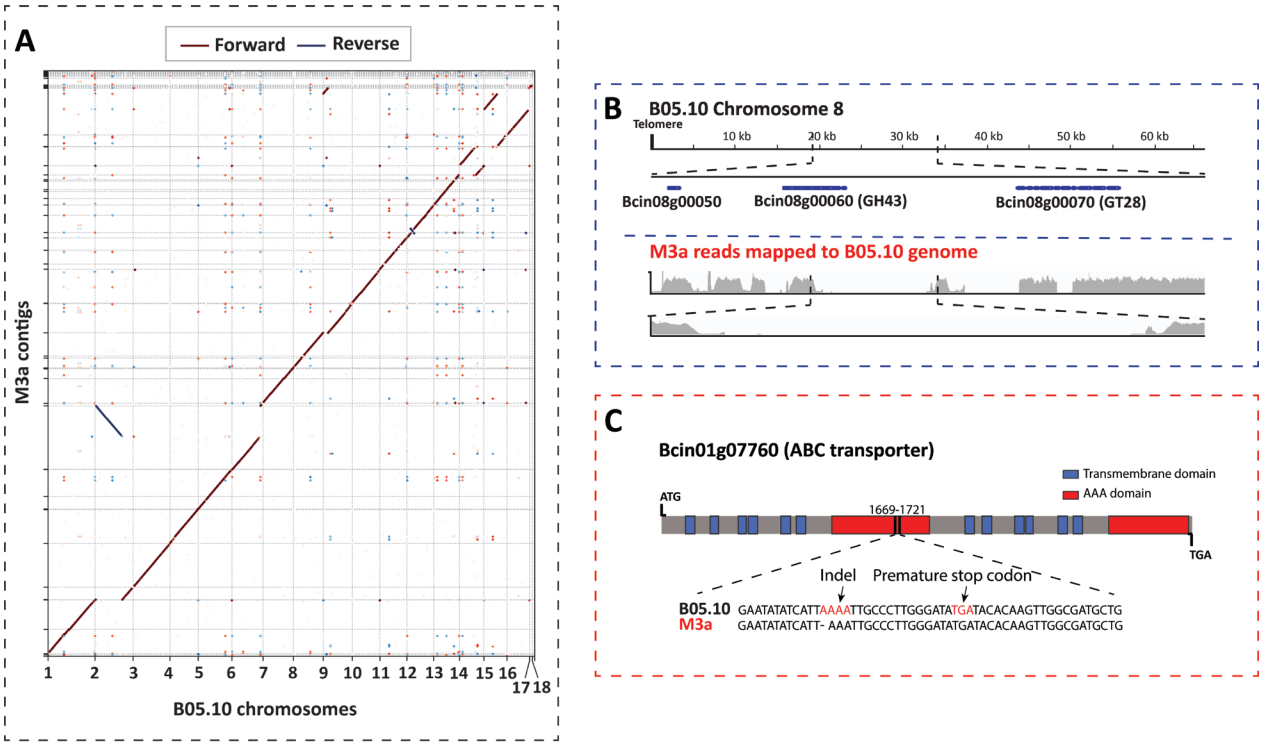


Figure 5. Characterization of genome differences between M3a and B05.10. Dot-plot of the filtered global alignment between M3a and B05.10 (A). Illustration of genomic difference between B05.10 and M3a regarding α-tomatine-responsive genes including *GH43*/*BcTom1* and *GT28a* (B) and *ABC*/*BcatrT* (C).

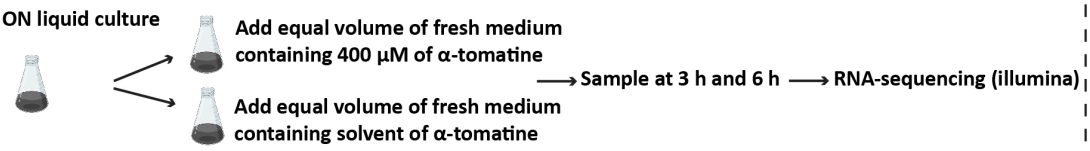
Identification of α-tomatine-responsive genes in *B. cinerea*

Exposure of fungi to growth inhibiting metabolites often results in the transcriptional upregulation of genes contributing to tolerance to these metabolites. This principle was exploited to identify *B. cinerea* genes that are induced in the presence of α-tomatine. The ON liquid culture of B05.10 was divided in two equal parts and one half was supplemented with fresh medium containing 200 µM α-tomatine, while the other half only received fresh medium. Samples were taken at 3 h and 6 h post supplementation, and RNA was extracted for sequencing (**Figure 6A**).

RNA-seq analysis revealed that α-tomatine treatment in B05.10 highly induced the expression of *GH43* and *GT28a* which are missing in M3a. Besides, another gene *GT28b* (Bcin09g02710) from GT28 family, as well as genes encoding membrane proteins, such as an ABC transporter BcatrT (Bcin01g07660) and four RTA1-like proteins: RTA1a (Bcin09g00800), RTA1b (Bcin06g00470), RTA1c (Bcin05g02090), RTA1d (Bcin02g0940) were also up-regulated after α-tomatine treatment (**Figure 6B**). Besides, these genes were expressed at low levels in the control treatment lacking α-tomatine (**Figure 6B**). We used reverse transcription quantitative polymerase chain reaction (RT-qPCR) to investigate the expression profiles of a subset of α-tomatine-responsive genes as well as *PEF1* over a more detailed time course after addition of

α-tomatine. As shown in **Figure 7A and Supplementary Figure S1**, in accordance with the RNA-seq results, all the genes showed low and stable transcript levels in the absence of α-tomatine and were induced in response to α-tomatine treatment with comparable induction levels (fold changes), except for *PEF1* whose mRNA abundance remained stable throughout all timepoints. Induction of all α-tomatine-inducible genes was observed at 30 mins and peaked between 3 h and 9h. Eventually, the transcript levels decreased at 24 h after supplementing the fungal culture with α-tomatine. The transcript levels of these genes were also analyzed in M3a. Remarkably, induction of α-tomatine-responsive genes was strongly compromised except for *RTA1a* which still displayed the high induction (**Figure 7B**).

A



B

Accession number	Pfam annotation	Fold change Tomatine/Mock		Mean TPM Mock			Mean TPM Tomatine	
		3 h	6 h	0 h	3 h	6 h	3 h	6 h
Bcin06g00470	RTA1 like protein	569.4	765.3	1	0	1	231	388
Bcin09g00800	RTA1 like protein	229.0	283.7	18	6	4	1332	1244
Bcin16g00006	Berberine and berberine like protein	199.2	96.3	0	0	0	11	9
Bcin08g00070	Glycosyltransferase family 28	199.1	202.1	2	1	1	120	131
Bcin08g00060	Glycosyl hydrolases family 43	107.3	124.4	9	4	5	481	576
Bcin01g07660	ABC transporter	107.2	85.9	1	1	0	62	43
Bcin09g02710	Glycosyltransferase family 28	44.7	75.8	1	0	0	11	18
Bcin06g06730	hypothetical protein	41.5	37.9	6	4	6	185	213
Bcin09g02720	Bacterial alpha-L-rhamnosidase	36.5	151.7	0	0	0	3	9
Bcin02g09400	RTA1 like protein	34.2	76.0	0	0	0	9	13

Mean TPM: 0 500 1000

Figure 6. Identification of α-tomatine-responsive genes in B05.10. Experimental design of the RNA-seq analysis (A) and list of highly induced genes (logFC>30) in B05.10 at 3 h and 5 h after addition of α-tomatine (B).

To better understand whether the increased transcript levels in B05.10 were specific to α-tomatine or resulted from a general response to membrane damage, we analyzed their expression profiles upon treatment with nystatin, a polyene antibiotic known to disrupt fungal membranes as well as another steroidal saponin digitonin originating from the foxglove plant *Digitalis purpurea*. Strikingly, expression levels of α-tomatine-responsive genes were strongly increased upon digitonin treatment but not after nystatin treatment (**Figure 7C and 7D**).

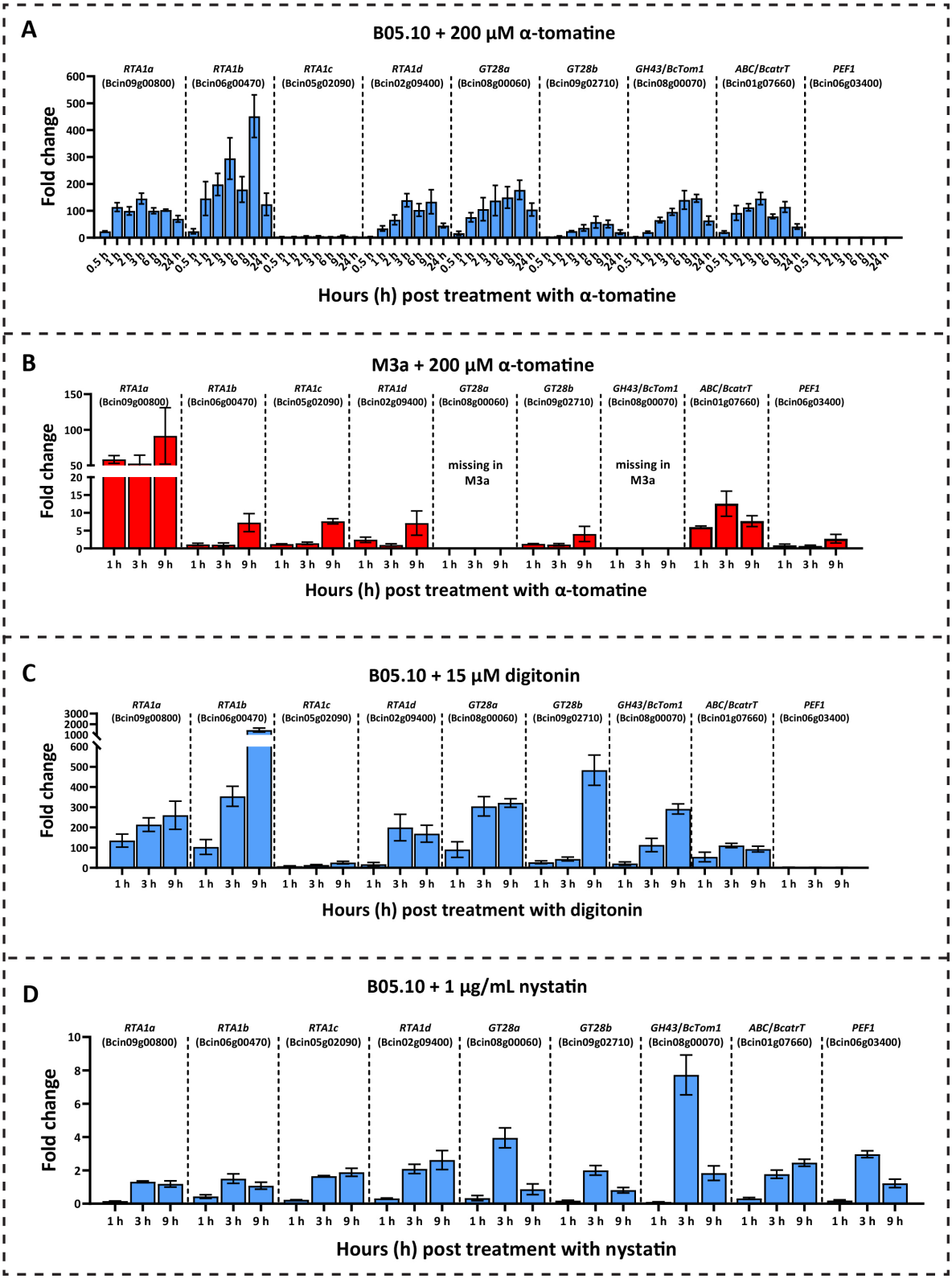


Figure 7. Relative expression of selected α-tomatine-responsive genes after addition of membrane-disrupting compounds in *B. cinerea* as compared to mock treatment. B05.10 treated with 200 µM α-tomatine (A); M3a treated with 200 µM α-tomatine (B); B05.10 treated with 15 µM digitonin (C); B05.10 treated with 1 µg/mL nystatin. RT-qPCR was performed using *SMT3* and *TUB* as reference genes. Error bars are SEM of three biological replicates.

Expression levels of α-tomatine-responsive genes during plant infection

We then analyzed the expression profile of α-tomatine-responsive genes during infection on MM leaves by RT-qPCR (**Figure 8A**). Most of these genes displayed low transcript levels in the early infection stages but strongly increased at later time points. The strong up-regulation was observed at 24 hours post inoculation (hpi) and coincided with the initiation of lesion expansion. *PEF1* transcript levels did not display strong elevation during tomato infection. By contrast, expression of α-tomatine-responsive genes were not up-regulated during infection on *N. benthamiana* or on *Phaseolus vulgaris* (**Figure 8B and 8C**)

Enzymatic detoxification of α-tomatine in *B. cinerea* is catalyzed by a GH43 protein

According to the carbohydrate-active enzymes database (CAZymes), proteins from the GH43 family have potential β-xylosidase activity which is in accordance with the mode of action of α-tomatine degradation in *B. cinerea* (Quidde et al., 1998; Lombard et al., 2014). We produced GH43 recombinant protein in *Escherichia coli* and tested it for tomatinase activity. After 1 h reaction with α-tomatine, putative degradation product β_1-tomatine was detected by LC-MS albeit a large amount of α-tomatine still remained and overlapped with β_1-tomatine in the chromatogram (**Figure 3**).To further confirm the function of the GH43 tomatinase candidate gene, the gene was deleted in B05.10, and was also overexpressed in M3a through a CRISPR/Cas9-mediated genome editing strategy. The B05.10 GH43 knockout (KO) mutant lost the ability to degrade α-tomatine, whereas the overexpression of the *GH43* gene conferred M3a tomatinase activity, as manifested by the detection of β_1-tomatine by LC-MS (**Figure 3**). These results collectively confirmed the exclusive role of the GH43 tomatinase in degrading α-tomatine into β_1-tomatine in *B. cinerea*, and the *GH43* gene will be designated as *BcTom*1 hereafter.

Phylogenetic analysis of GH43/BcTom1

Phylogenetic analysis of the BcTom1 protein sequence indicated that within the Sclerotiniaceae, this gene is only present in the genus *Botrytis* (**Supplementary Figure S2**). Specifically, we only identified orthologs in *B. aclada* (BACL_015g04100) and *B. calthae* (BCAL_0134g00010) which share 85% and 87% protein sequence identity, respectively (Valero-Jiménez et al., 2019; Valero-Jiménez., 2020). Moreover, orthologs of BcTom1 were identified in more distantly related plant pathogenic fungi including *Stemphylium lycopersici* (KNG52549) and *Alternaria arborescens* (RYN15858.1) that can both infect tomato (**Figure 9**).

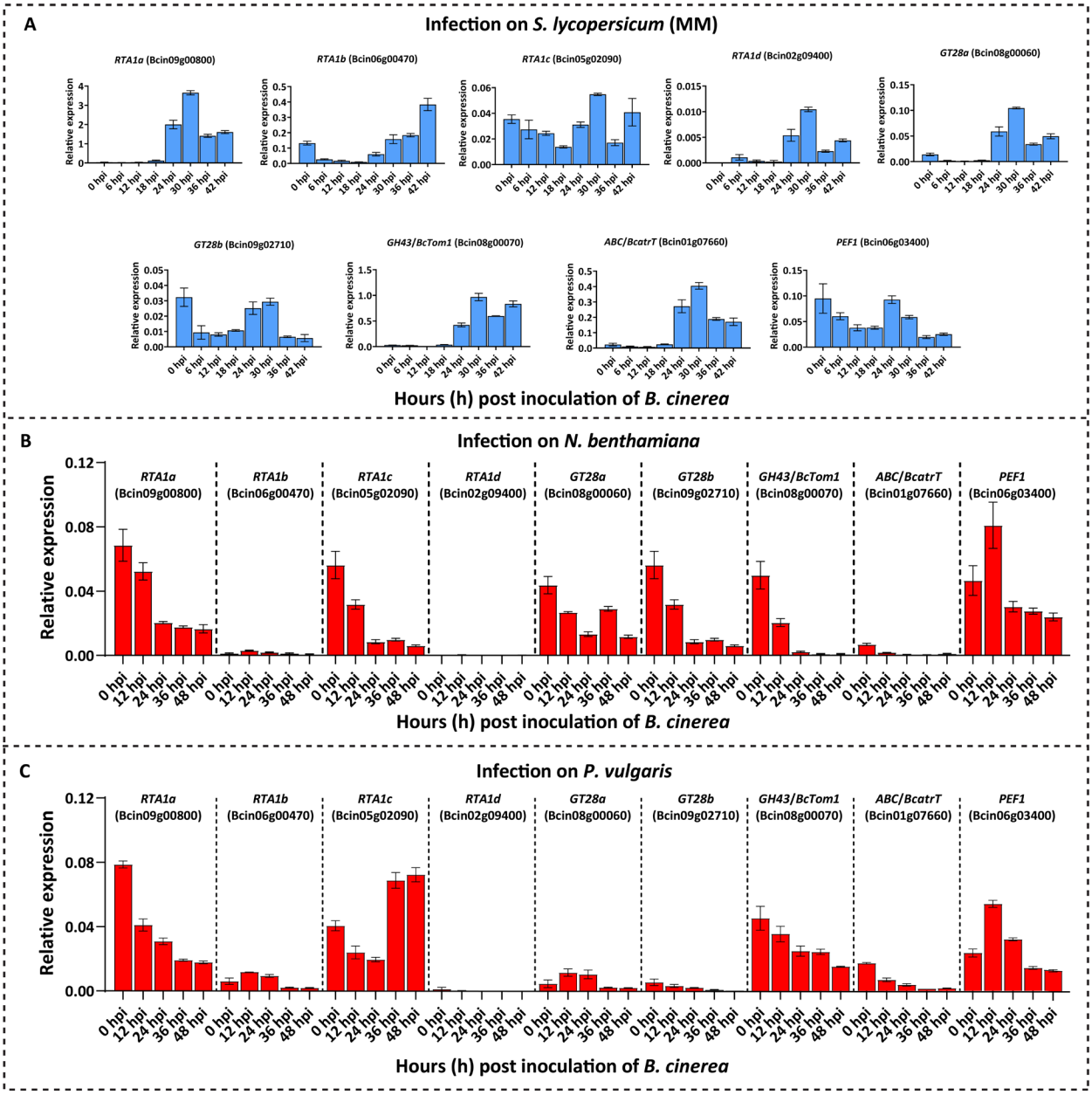


Figure 8. Analysis of relative expression of selected α-tomatine-responsive genes after inoculation on different plants**.** Infection on MM leaves (A), *N. benthamiana* leaves (B) or *P. vulgaris* leaves (C) by RT-qPCR using *SMT3* and *TUB* as reference genes. Error bars are SEM of three biological replicates**.**

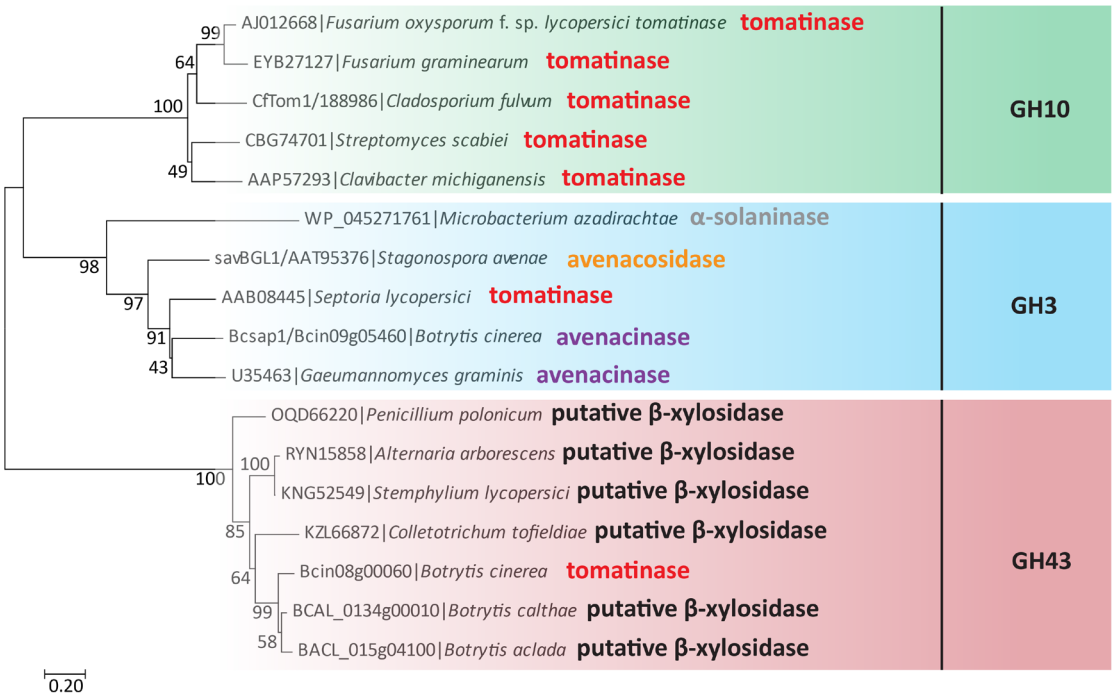


Figure 9. Phylogenetic relationships between reported tomatinases and other saponin-deglycosylating hydrolases from GH3, GH10 and GH43 families. Phylogenetic tree was constructed using minimum evolution algorithm (1000 bootstraps) based on the alignment of amino acid sequences.

α-Tomatine-degrading enzymes contribute to tolerance to α-tomatine and virulence on tomato

The *BcTom1*-KO mutants displayed increased sensitivity to α-tomatine on agar plates, manifested as slower radial growth than the wild type (WT) B05.10 (**Figure 10**) as well as reduced virulence on tomato (**Figure 11**). To gain a more complete view of the involvement of tomatinase activity in α-tomatine tolerance and plant infection, we overexpressed the heterologous tomatinase genes *CfTom1* (GH10) from *C. fulvum* and *SlTom1* (GH3) from *S. lycopersici* in M3a, respectively. The overexpression of the two types of tomatinase genes conferred on M3a the corresponding ability to degrade α-tomatine, shown by the detection of the correct breakdown products deduced from their reported catalytic activity: tomatidine and β_2-tomatine, respectively (**Figure 3**). Moreover, expression of heterologous tomatinases in M3a resulted in a higher proportion of expanding lesions (disease incidence) upon inoculation on tomato and it increased the tolerance to α-tomatine (**Figures 11 and 12**). In addition, inoculation of M3a transformants overexpressing different types of tomatinase gene or B05.10 *BcTom1*-KO mutants on *N. benthamiana* did not exhibit differences in lesion sizes as compared with wild type recipients, except for M3a expressing *SlTom1*-OE, which formed smaller lesions (**Figure 13**).

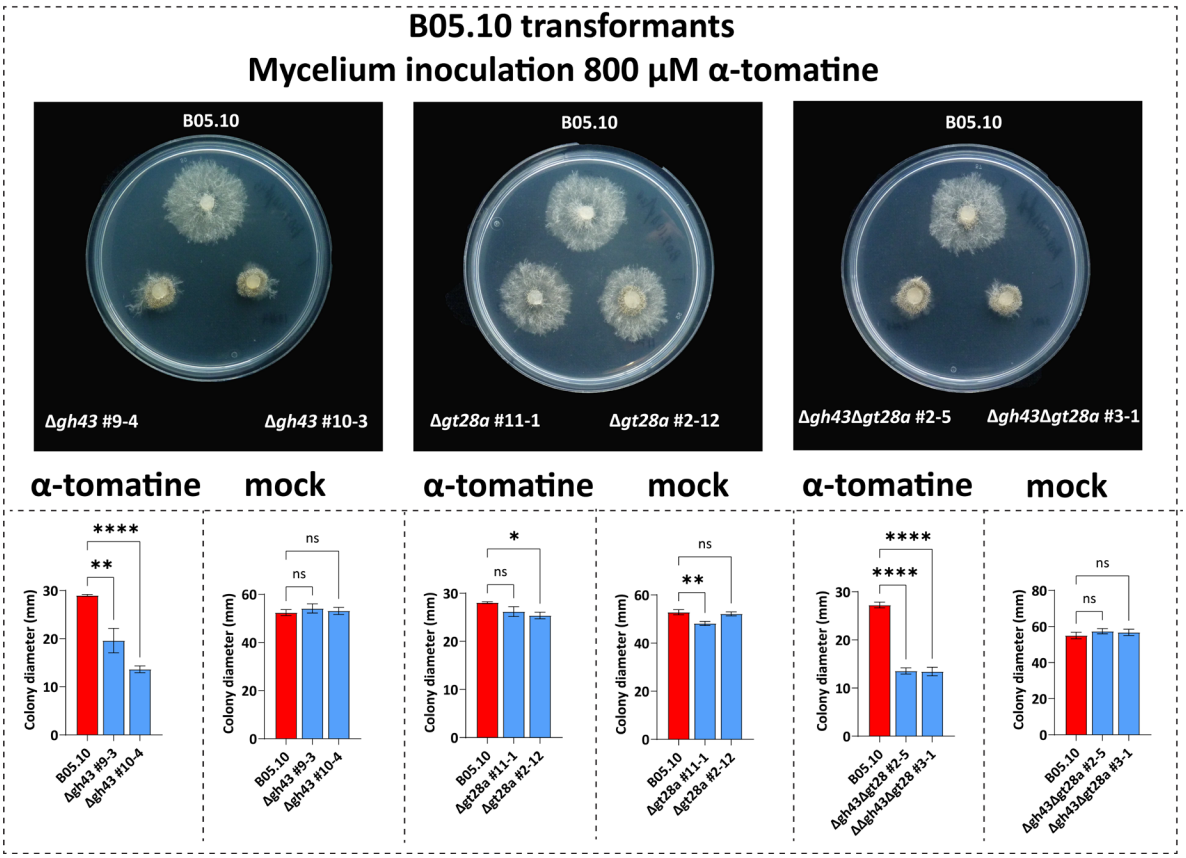


Figure 10. Sensitivity test of B05.10 transformants against α-tomatine. Fungal colony growth were from 5 mm mycelium agars inoculated on GB5-10 mM sucrose-10 mM phosphate plates containing 800 µM α-tomatine. Colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01; **** p-value <0.0001). ns indicates no significant difference.

The GT28 glycosyl transferase contributes to tolerance to α-tomatine and is required for full virulence on tomato

M3a also lacks the *GT28a* gene that resides next to the *BcTom*1, close to the telomeric region of Chr 8 (**Figure 5**). The B05.10 *GT28a* KO mutants displayed reduced virulence on tomato but were not affected in resistance to α-tomatine (**Figures 11 and 12**). Overexpression of GT28a in M3a resulted in larger colony diameters on α-tomatine plates although the resistance-promoting effect was not as strong as the overexpression of either of the three tomatinase genes, *GH43*, *CfTom1* or *SlTom1* (**Figure 12**). Moreover, neither overexpression *GT28a* in M3a nor deletion of GT28a in B05.10 affected their virulence on *N. benthamiana* (**Figure 13**). Besides, we also investigate the role of GT28a in tolerance against polyene antibiotic nystatin which can also can membrane disruption in fungi. However, neither deletion of GT28a in B05.10 nor OE of GT28a in M3a affected their sensitivity to nystatin (**Figure 14**).

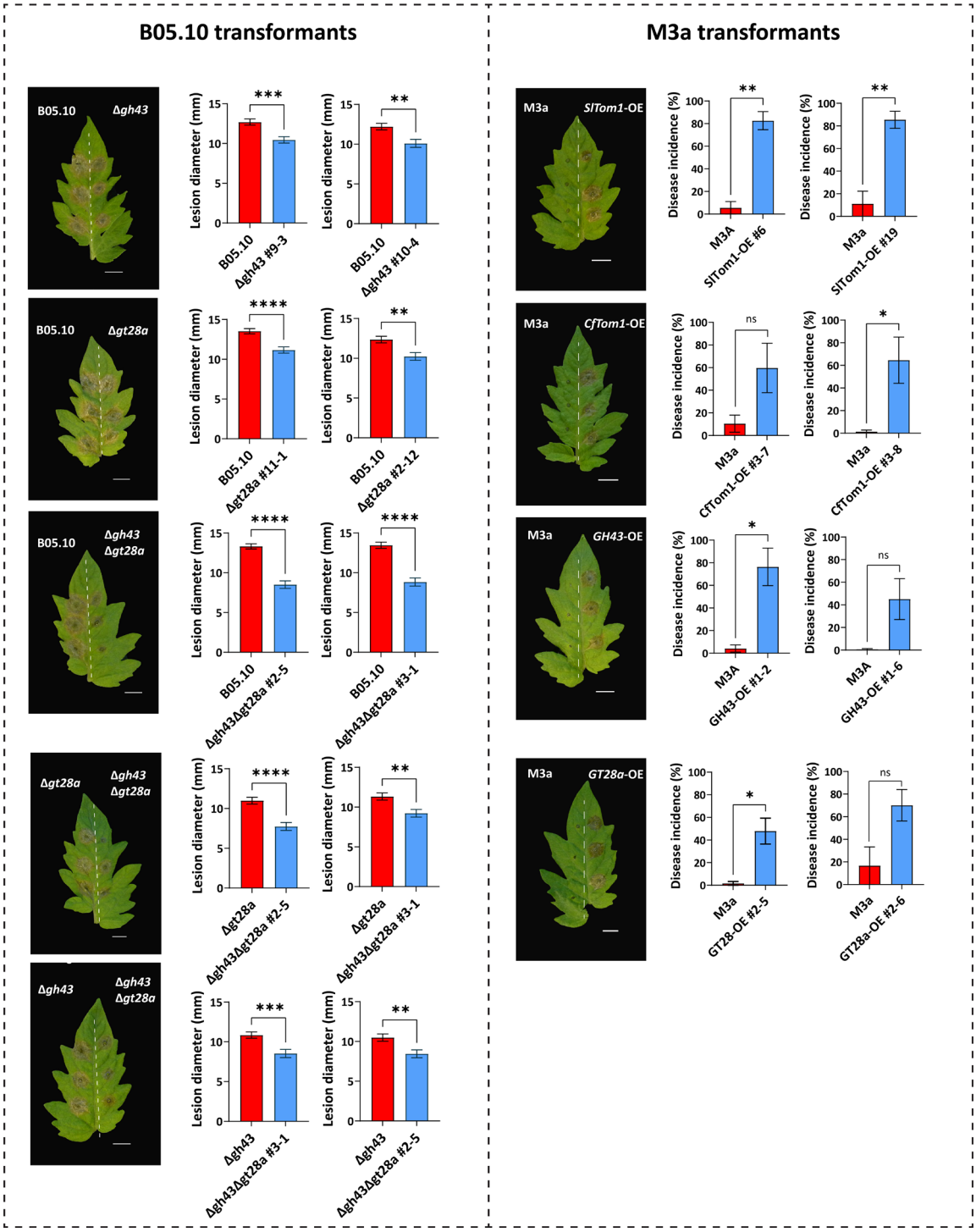


Figure 11. Infection of *B. cinerea* transformants on tomato leaves. WT strains were inoculated on the left side of the vein (as depicted by the dashed line). Transformant strains were inoculated on the right side of the vein. Scale bar indicates 1 cm. Error bars are SEM of at least two independent inoculation assays. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01; *** p-value <0.005; **** p-value <0.0001). ns indicates no significant difference.

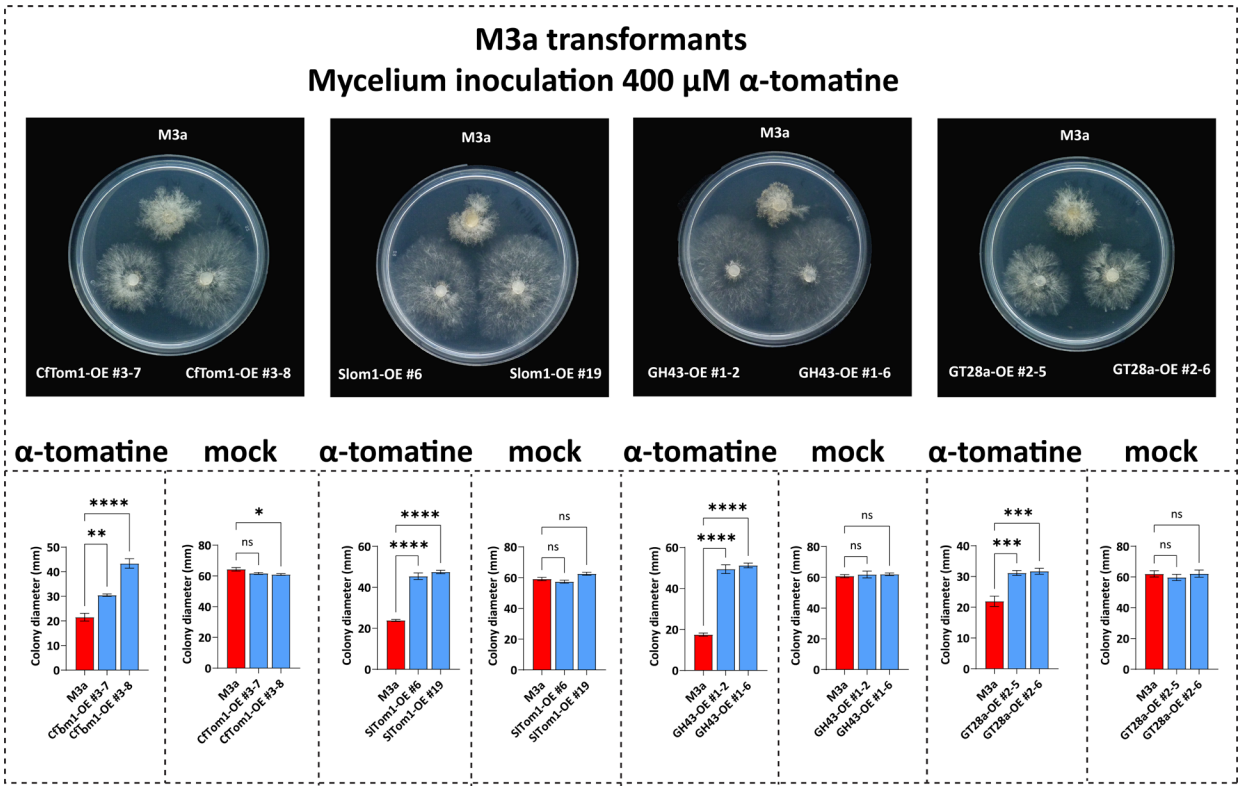


Figure 12. Sensitivity test of M3a transformants against α-tomatine. Fungal colony growth were from 5 mm mycelium agars inoculated on GB5-10 mM sucrose-10 mM phosphate plates containing 400 µM α-tomatine. Colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01; *** p-value <0.005; **** p-value <0.0001). ns indicates no significant difference.

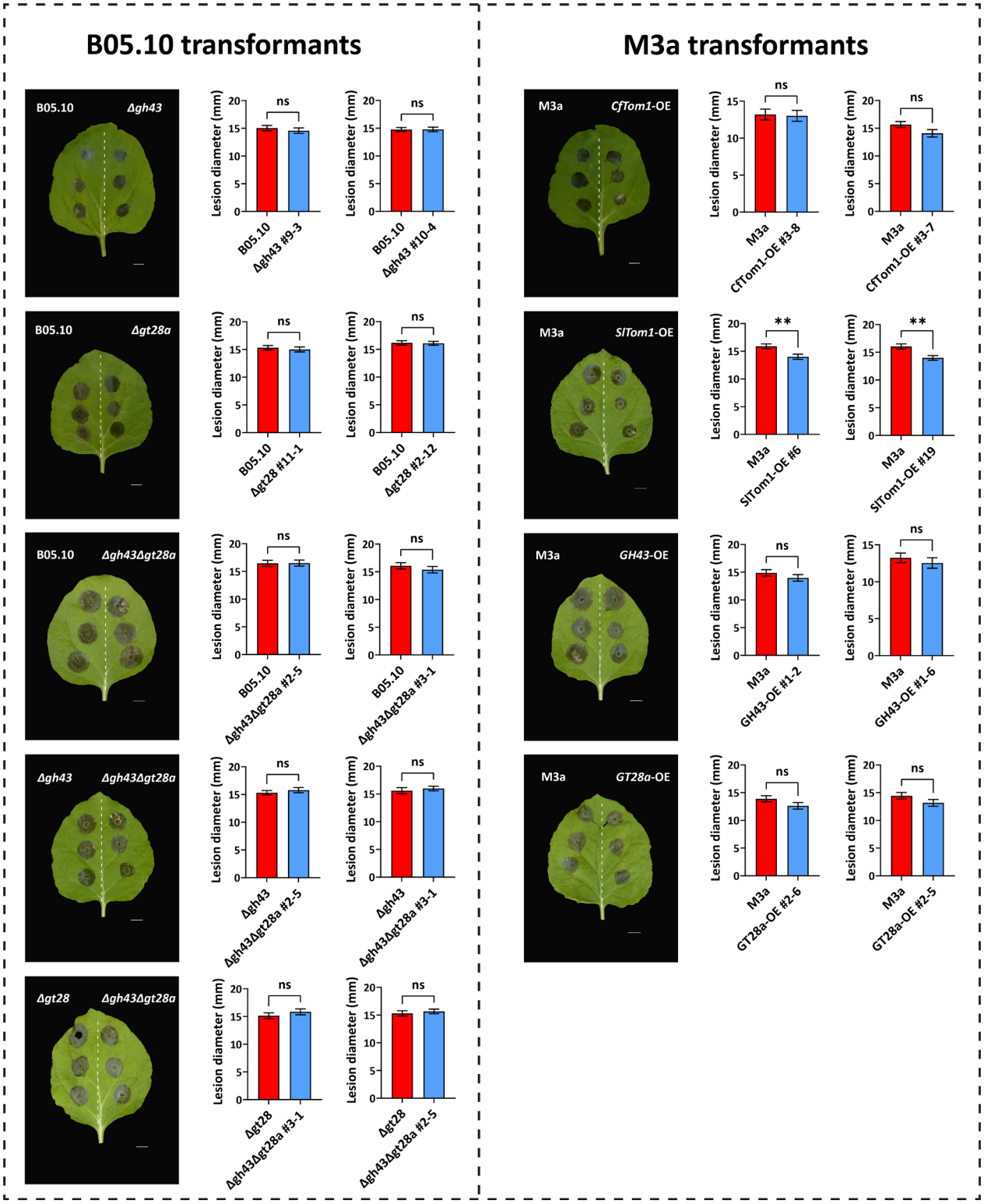


Figure 13. Virulence of *B. cinerea* transformants on *N. benthamiana*. WT strains were inoculated on the left side of the vein (as depicted by the dashed line). Transformant strains were inoculated on the right side of the vein. Scale bar indicates 1 cm. Error bars are SEM of at least two independent inoculation assays. The asterisks represent statistically significant differences determined by the student's t-test (** p-value < 0.01). ns indicates no significant difference.

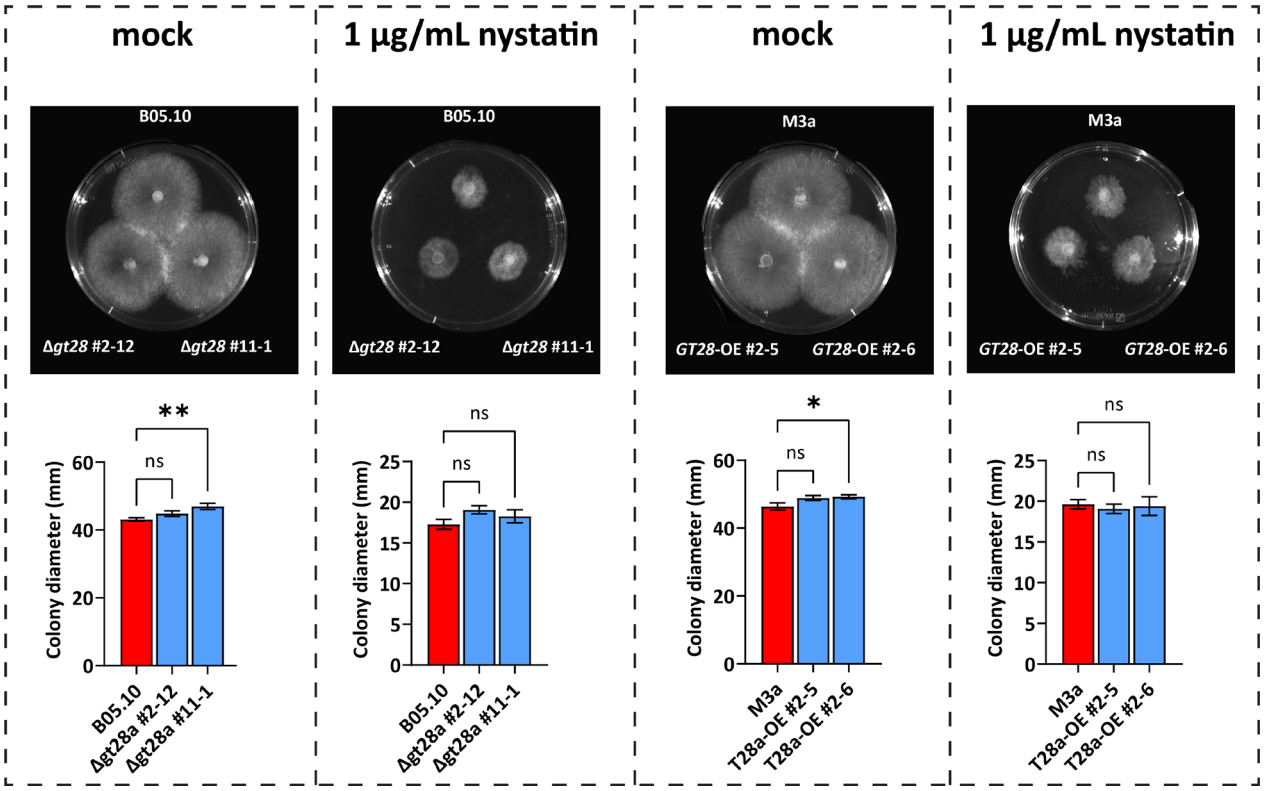


Figure 14. Sensitivity test of *B. cinerea* transformants against nystatin. Fungal colony growth were from 5 mm mycelium agars inoculated on MEA plates containing 1 µg/mL nystatin. Colony diameters were measured at 2 dpi. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01). ns indicates no significant difference.

GT28a predominantly localized to the cytosol and can be recruited to the membrane upon treatment with membrane-disrupting compounds

To determine the subcellular localization of GT28a, GT28a-GFP fusion protein was expressed in B05.10 under the control of constitutive promoter. In complete medium without α-tomatine, the GFP fluorescence signal was homogenously distributed in the hyphae indicating GT28a is predominantly a cytoplasmic protein. After treatment addition of α-tomatine and nystatin to the fungal culture, the GFP signal accumulated more intensely at hyphal tips (**Figure 15**).

The recruitment of GT28a-GFP to the membrane is independent of Ca^{2+} and PEF1

The recruitment of GT28a-GFP to the membrane after α-tomatine or nystatin treatment were unaffected in the presence or absence of Ca^{2+} (**Figure 15**). We also generated the *PEF1*-KO mutant in the background of B05.10 GT28a-GFP-OE transformants to investigate the role of PEF1 in the membrane recruitment of GT28a-GFP in response to α-tomatine or nystatin treatment. The mobilization of GT28a-GFP to the membrane of hyphae tips visible as GFP fluorescence signal was still observed with high incidence in the absence of PEF1 (**Figure 16**).

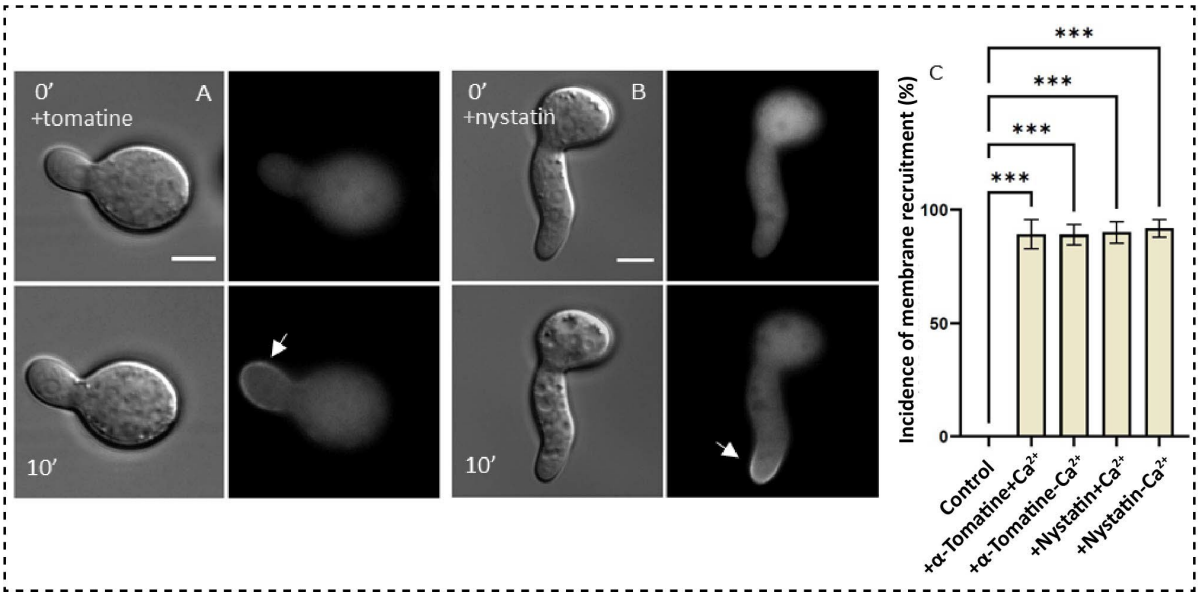


Figure 15. Subcellular localization of GT28a-GFP in the presence/absence of Ca^{2+}. Images of *B. cinerea* germinated spores taken at 10 minutes (mins) after treatment with 193 µM α-tomatine (A) or 0.05 mg/mL nystatin (B); Incidence of GT28a-GFP recruitment to the membrane after treatment with α-tomatine or nystatin in the presence/absence of Ca^{2+} (C). Error bars indicate the SD calculated from three independent experiments (n = 100 each). The asterisks represent statistically significant differences determined by the student's t-test (*** p-value <0.005). The scale bar indicates 5 µm.

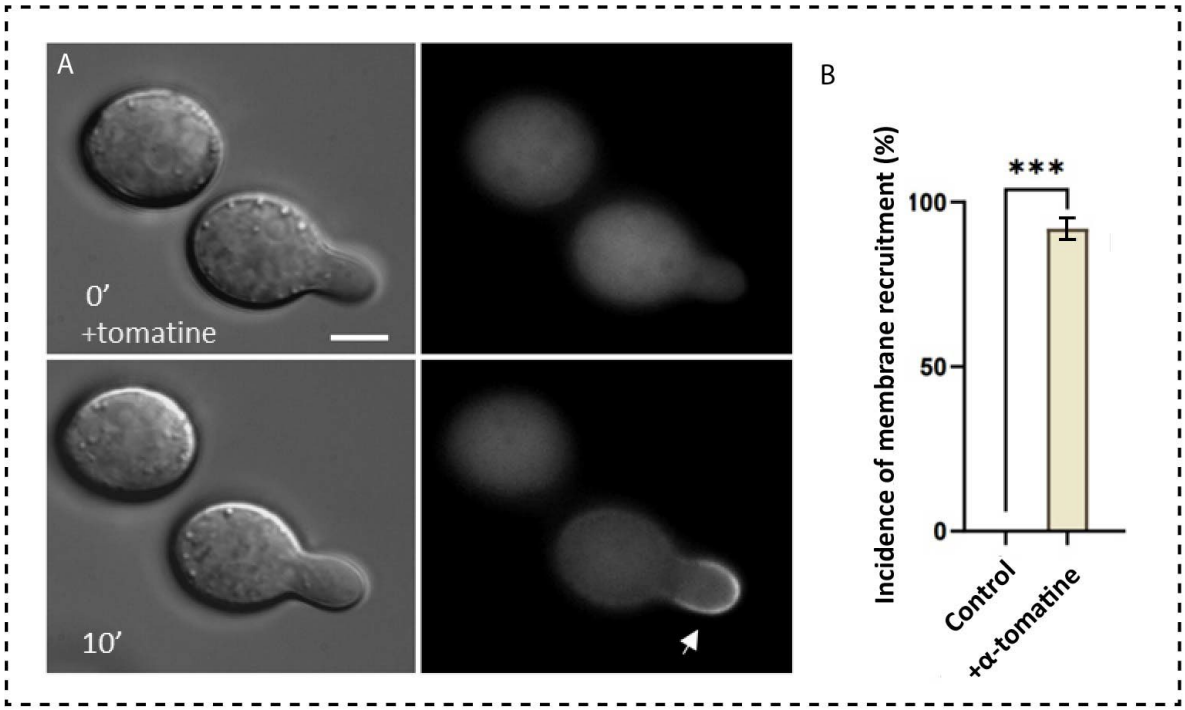


Figure 16. Subcellular localization of GT28a-GFP in *PEF1* KO mutant. Images of *B. cinerea* germinated spores taken at 10 minutes (mins) after treatment with 190 µM α-tomatine (A); Incidence of GT28a-GFP recruitment to the membrane in the B05.10 GT28a-GFP-OE *PFE1* KO transformants. Error bars indicate the standard deviation (SD) calculated from three independent experiments (n = 100 each). The asterisks represent statistically significant differences determined by the student's t-test (*** p-value <0.005). The scale bar is 5 µm.

Discussion

B. cinerea was reported to degrade α-tomatine by a different mechanism than any other microbe, by removing of the terminal xylose and releasing β_1-tomatine (Quidde et al., 1998; You and van Kan, 2021).This made the identification of the gene encoding *B. cinerea* tomatinase challenging as it was unlikely to be homologous to other microbial tomatinase genes. Besides, the analysis of culture fluids from *B. cinerea* grown in presence of α-tomatine identified β_1-tomatine as the only breakdown product and thus excluded the presence of other type of tomatinase activity in *B. cinerea*. Indeed, the B05.10 genome does not contain homologs to GH10 tomatinase genes, whereas the gene *Bcsap*1, which has high sequence similarity to *S. lycopersici* GH3 tomatinase has been reported to be irrelevant to the degradation of α-tomatine (Quidde et al., 1999). We identified the gene encoding tomatinase based on its transcriptional upregulation, as induction of tomatinase activity or of tomatinase gene expression by α-tomatine have been reported in several tomato pathogens (Ökmen et al., 2013; Lairini et al., 1997). The up-regulation of *BcTom1* in response to α-tomatine is in accordance with the observation of increased tomatinase activity reported in B05.10 in the previous study (Quidde et al., 1998). Moreover, the *BcTom1* gene appeared to be absent from the genome of isolate M3a, which was sampled from grape and unable to degrade α-tomatine.

Previous studies have shown that degradation of saponins can determine the host specificity. Mutants of the oat fungal pathogen *Gaeumannomyces graminis* unable to degrade avenacin A-1 became more sensitive to avenacins and could no longer infect oat roots. However, the mutants remained pathogenic on wheat plants which do not contain avenacins (Bowyer et al., 1995). A similar phenomenon was observed with the tomatinase-deficient isolate M3a collected from grapes. Under certain inoculation conditions, M3a failed to develop expanding lesions on tomato leaves which suggests that the infection was fully stopped by α-tomatine presumably due to failure of M3a to counteract the toxic effects of α-tomatine. The overexpression of three distinct types of tomatinase genes (encoding GH3, GH10 or GH43 family enzymes) restored the ability to colonize tomato in M3a which highlights the importance of saponin-degrading activities for infecting host plants.

The deletion of either BcTom1 or GT28a in B05.10 significantly reduced lesion sizes on tomato and the double KO mutants exhibited even stronger reduction in virulence. Nevertheless, the BcTom1/GT28a double KO mutants were still able to cause expanding lesions on tomato under the inoculation condition that prohibited expanding lesion development by M3a. This observation suggests the presence of additional mechanisms for tolerance to α-tomatine in B05.10. The whole-genome comparison of B05.10

and M3a revealed, besides the absence of GH43/BcTom1 and GT28 in M3a, more than 100 genes with sequence polymorphisms that result in amino acid substitutions or premature stop codons. Follow-up studies on this set of genes are needed for the identification of additional mechanisms for tolerance to α-tomatine.

The fact that the tomatinase-deficient grape isolate M3a lacks both the GH43/BcTom1 and GT28a is consistent with a recent genomic comparison between *B. cinerea* field isolates collected from tomato and grape (Mercier et al., 2021). The region encompassing four genes, including *BcTom*1 and *GT*28a, appeared to be present in three copies in all the sequenced tomato isolates, while the grape isolates analyzed frequently lacked either *BcTom*1 or *GT*28a, or both. Combined with our observation of the significant role of these two genes in virulence on tomato but not on *N. benthamiana*, it strongly suggests that *BcTom*1 and *GT*28a contribute to host adaption to tomato due to their importance for tolerance to α-tomatine.

Some studies have revealed that degradation of saponins not only results in glycoalkaloid detoxification, but can also modulate host immune responses via the α-tomatine hydrolytic breakdown products (You and van Kan, 2021). The exposure of *N. benthamiana* to *S. lycopersici* tomatinase led to the production of an unknown metabolite that could mediate the suppression of plant defense responses and was essential for pathogenicity of *S. lycopersici* (Bouarab et al., 2002). However, in our study, *B. cinerea* M3a overexpressing the *SlTom* exhibited slightly smaller lesion sizes compared to the WT M3a on *N. benthamiana*. This observation further underlines the importance of dissection of the suppression of defense responses and host cell death in different pathosystems. For instance, the *S. lycopersici* tomatinase mutant elicited strong localized hypersensitive cell death in the guard cells which prevented the fungus from entering the host tissues. By contrast, *B. cinerea* infection does not require entry through stomata and can even benefit from host cell death (Veloso and van Kan,2018). Thus the inferred suppression of defense response and plant cell death in *N. benthamiana* might play a positive role in resistance against *B. cinerea*.

Membrane transporter proteins can serve as efflux pumps for various compounds and especially ABC transporters are well-recognized to confer tolerance against antimicrobial compounds, for instance, the phytoalexins resveratrol from grape (Schoonbeek et a., 2001) and camalexin from Arabidopsis (Stefanato et al., 2009), as well as azole fungicides which inhibit ergosterol biosynthesis (Stergiopoulos et al., 2002). However, there is a lack of studies investigating the role of membrane transporters in tolerance to membrane-permeating compounds such as polyene antibiotics and saponins. We identified a previously uncharacterized ATP-binding cassette (ABC) transporter gene *BcatrT* whose expression can be rapidly induced by α-tomatine . Remarkably, the ABC transporter gene in B05.10 contains a premature stop codon

in the ATPase domain as a result of the insertion of a single nucleotide, which leads to a frameshift and results in the production of a truncated protein defective in domains critical for the transporter function. This ABC transporter is very likely to be a pseudogene in B05.10 albeit its transcript levels are strongly induced by α-tomatine. By contrast, the gene model in M3a has a functional open reading frame. Experiments to examine the possible role of this ABC transporter in α-tomatine efflux and in virulence of *B. cinerea* on tomato are ongoing.

The damage of the membrane can lead to downstream signal transduction. For instance, the recruitment of PEF1 in *N. crassa* in response to α-tomatine and nystatin treatment has been demonstrated to be partially dependent on the influx of extracellular Ca^{2+} into the fungal cells (Schumann et al., 2019). However, the recruitment of GT28a-GFP to the membrane was independent of Ca^{2+} and did not require PEF1 to be mobilized to the membrane after α-tomatine treatment. Among the proteins that may participate in GT28a recruitment to the membrane are annexins, but it will require further studies to unravel this process.

Treatment of B05.10 with nystatin did not strongly induce the expression of α-tomatine-responsive genes, implying that the transcriptional response is presumably specific to the molecular structure of α-tomatine but not due to its membrane-disrupting effect. Notably, digitonin which is a steroidal saponin having five sugar residues in the side chain can also strongly up-regulate all α-tomatine-responsive genes. This is consistent with the observation by Quidde et al. (1998) that tomatinase activity in B05.10 can be induced by digitonin. Another interesting observation is that the induction of α-tomatine-responsive genes in M3a upon α-tomatine treatment was severely compromised. Therefore, M3a as a grape isolate not only lost the *GH43*/*BcTom1* and *GT28a* genes which are important for tolerance to α-tomatine and required for full virulence on tomato, but was also partially defective in the transcriptional response to α-tomatine. The genome comparison of B05.10 and M3a revealed a gene (Bcin12g01000) encoding a putative protein-serine/threonine kinase that was absent in M3a and therefore might be involved in the up-regulation of α-tomatine-responsive genes. This hypothesis will be further explored in future studies.

Materials and methods

Fungal growth and transformation

For spore production, 0.5 µL glycerol-stock of $1x10^7$/mL spores were plated on Malt Extract Agar (MEA) plates and incubated in the dark at 20°C. When the mycelium reached the edge of the petri dishes, the plates were exposed to near-UV for 24h to induce sporulation. Afterwards, the plates were transferred back to the dark. After 5-10 days spores were harvested and counted with a haemocytometer and re-suspended to a final concentration of $1x10^7$/mL.

CRISPR/Cas9 mediated transformation of *B. cinerea* was performed according to Leisen et al. (2020). Single Guide RNAs (sgRNAs) targeting the gene of interest were synthesized prior to the tranformation. For overexpression, gene loci of the nitrate reductase (BcniaD; Bcin07g01270) and the nitrite reductase (BcniiA; Bcin01g05790) were used for the targeted integration of the overexpression cassette generated in pNDH-OGG and pNAN-OGG vectors as described in Schumacher (2012). BlastN from the Ensembl platform was used to check the specificity of the sgRNA in the *B. cinerea* genome (Howe et al., 2020; van Kan et al., 2017). The donor DNA comprising selection marker genes templates were amplified by PCR using primers containing 60 bp homologous recombination region.

For transformation, *B. cinerea* protoplasts were generated by incubating the *B. cinerea* overnight liquid culture with 1% Glucanex (Sigma Aldrich) and 0.1% Yatalase (Takara) in KCl phosphate buffer solution. Then the protoplasts were transformed with Cas9/sgRNA ribonucleoprotein complexes (RNP) and DNA donor template mediated by 60% PEG3350 solution. After 3 to 5 days, resistant colonies were transferred to MEA plates containing the corresponding antibiotic and colonies were grown for genotyping. PCR reaction using flanking primers spanning the target genes was used for genotyping. The PCR products with expected size were sequenced to confirm the transformation. At least two independent homokaryotic transformants were used for further characterization.

Plant growth and infection assays

Tomato *S. lycopersicum* cv. Moneymaker (MM), *N. benthamiana* plants and French bean *Phaseolus vulgaris* were grown in the greenhouse at 21/19 °C (day/night) temperatures with 16/8 h light/darkness rhythm and a relative humidity of 60-80%. Fully expanded leaves from 5 to 6 week-old tomato plants were detached and used for inoculation under lab conditions. Leaves from 4 to 5 week-old *N. benthamiana* plants or 2-week-old *P. vulgaris* were used for *B. cinerea* infection assays.

For tomato inoculation, *B. cinerea* spores were diluted in 3 g/L Gamborg's B5 (Duchefa BV) minimal medium supplemented with 10 mM sucrose and 10 mM potassium phosphate and adjusted to pH 6.0 at

a final concentration of $1x10^6$/mL as described in Benito et al. (1998). For inoculation on *N. benthamiana*, *B. cinerea* spores were diluted in potato dextrose broth (PDB) (12 g/L) at a concentration of $1x10^6$/mL. Adaxial side of the leaf was inoculated with 3 droplets of 2 µL inoculum (2000 spores). The recipient strain was inoculated on the left side of the leaf while the related transformants were inoculated on the right side. At 3 dpi, inoculated leaves where photographed and lesion sizes were measured with a digital caliper.

Gene expression analysis of α-tomatine-responsive genes in *B. cinerea*

B. cinerea spores were inoculated in liquid medium containing 3 g/L GB5 minimal medium, 100 mM fructose, 10 mM potassium phosphate, 0.5% yeast extract and adjusted to pH 5.5 at a final concentration of $1x10^6$/mL. After overnight incubation at 20 °C 120 RPM, half of the liquid culture was supplemented with α-tomatine (TCI Europe) and the other half was supplemented with solvent used to dissolve α-tomatine (pure methanol + 0.5% formic acid). *B. cinerea* samples were collected at 0 h, 3 h and 6 h after α-tomatine/mock treatment and used for mRNA isolation. RNA-seq was carried out at Beijing Genomics Institute (BGI), Shenzhen, China. The Illumina reads were mapped to the B05.10 genome published by van Kan et al. (2017). The gene expression level was manifested as transcripts per million (TPM).

Induction of α-tomatine-responsive genes were further validated in B05.10 and M3a as described above, with more time points (B05.10: 0.5 h, 1 h, 2 h 3 h, 6 h, 9 h and 24 h; M3a: 1 h, 3 h and 9 h) by RT-qPCR. Besides, nystatin (Sigma Aldrich) treatment (1 mg/mL) and digitonin (Carl Roth) treatment were carried out on B05.10 in a similar way and sampled at 1 h, 3h and 9 h after treatment. Expression of α-tomatine-responsive genes was analyzed by RT-qPCR.

Expression of α-tomatine-responsive genes was also investigated during tomato and *N. benthamiana* infection at different timepoints (tomato: 0 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 30 hpi, 36 hpi and 43 hpi; *N. benthamiana*: 0 hpi, 12 hpi, 24 hpi, 36 hpi and 48 hpi).

Sensitivity test to membrane-disrupting compounds using plate assays

To test the sensitivity of *B. cinerea* transformants to α-tomatine, both spores and mycelium were plated on 15 mL GB5 minimal medium plates containing varying amounts of α-tomatine (B05.10 spores 600 µM; B05.10 mycelium 800 µM; M3A spores 200 µM; M3A mycelium 400 µM). For mycelium inoculation, Mycelium plugs of 5 mm were taken from the actively growing edge of *B. cinerea* colony growing on MEA plates and placed on the α-tomatine containing plates with the mycelium facing the agar. For spore inoculations, spore concentrations were adjusted to $1x10^6$/mL and 2 µL droplets (2000 spores) were inoculated on the plate. Each plate contains one droplet spot of a WT strain (B05.10 or M3A) and two

droplet spores from two independent transformants, respectively and 5 replicates were performed. Plates were incubated in the dark at 20°C and colony diameters were measured at 3 dpi.
For testing the sensitivity to nystatin, only mycelium plugs were inoculated on MEA plates containing 1 µg nystatin/mL. Inoculations and measurements were performed as described for the α-tomatine plate assay. Colony diameters were measured at 2 dpi.

M3a genome sequencing, assembly and comparative genomics analysis with B05.10

Genomic DNA of M3a was extracted and sequenced by Illumina and nanopore sequencing. The Illumina and nanopore reads were combined into an initial assembly of M3a using the MaSuRCA genome assembler (Zimin et al., 2013). The genomes of B05.10 and M3a were compared using four different methods. Firstly, a global alignment between the two genomes was produced using the NUCmer algorithm (Stefan et al., 2004). Secondly, an alignment-free comparison was performed using the shortest unique substrings (shustring) algorithm (Haubold et al., 2005). Thirdly, short reads obtained from Illumina sequencing on the M3a genome were aligned to the B05.10 genome using the BWA-MEM algorithm. Fourthly, long reads obtained from Oxford Nanopore sequencing on the M3a genome were aligned to the B05.10 genome using the Minimap2 algorithm (Li, 2018). Based on the Illumina reads alignment, Single Nucleotide Polymorphisms (SNPs) and small insertions and deletions (indels) were mapped using the FreeBayes algorithm (Garrison and Marth, 2012). The snpEff algorithm was used to predict the effect of the SNPs on the produced proteins (Cingolani et al., 2012).

α-Tomatine and digitonin degradation assay

Recombinant protein of BcTom1 was produced in *E. coli* strain BL21 using the pET-15b expression system (Abedi et al., 2012). 12.5 µg pure protein of BcTom1 was incubated with 25 µM of α-tomatine for 1 h and the conversion products were detected by LC-MS. WT strains (B05.10 and M3a) and their transformants were grown in liquid culture as described above and were incubated with 200 µM α-tomatine/100 µM digitonin for 9 h. Conversion products in the supernatant were detected by LC-MS.

General microscopy and analysis

Fungal cells were observed on a Zeiss Observer 2.1 microscope using Nomarski optics with a Plan-Neofluar 100×/1.30 oil immersion objective (420493-9900) with CoolLED pE4000 as a light source for fluorescence microscopy. Images were captured with a PCO Edge 5.5 Gold (16 bit) camera and analyzed using ImageJ.

Sample preparation for microscopy and quantitative recruitment assay

To analyse GT28a-GFP recruitment in response to the anti-fungal drugs nystatin and α-tomatine, 5 µL of $2x10^7$ spores were incubated in 150 mL liquid mineral medium in Ibidi eight-well µ-slides (Sigma-Aldrich) for 5-6 h at 20°C. After imaging the untreated cells, 100 µL of 193 µM α-tomatine solution was added. Similarly, for nystatin 10 µL of 0.05 mg/mL was added and mixed by pipetting, and incubated for 5-10 min before analysis by Nomarski and fluorescence microscopy. For quantitative assays, 100 germlings were tested for GT28a-GFP recruitment. Each test was independently repeated two more times with multiple technical replicates.

Sterol staining

To stain membrane sterols in germlings, 100 µL of 100 µg/mL of filipin III solution in 1% (v/v) DMSO were added to the germlings grown in Ibidi eight-well µ-slides as mentioned above. Cells were incubated for 20 mins and analyzed by fluorescence microscopy using a DAPI filter setup.

Acknowledgements

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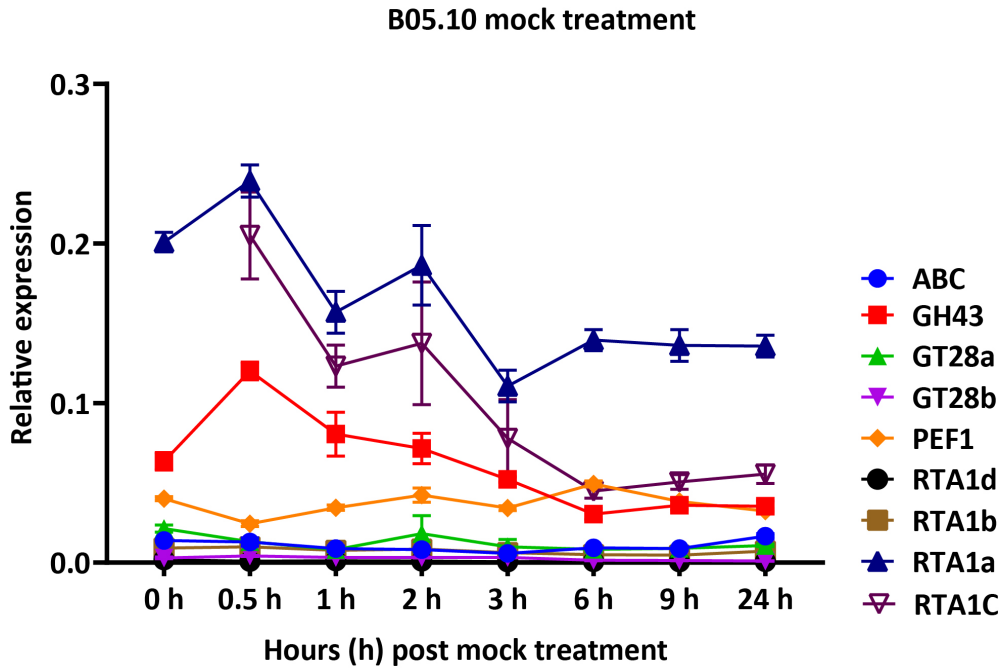
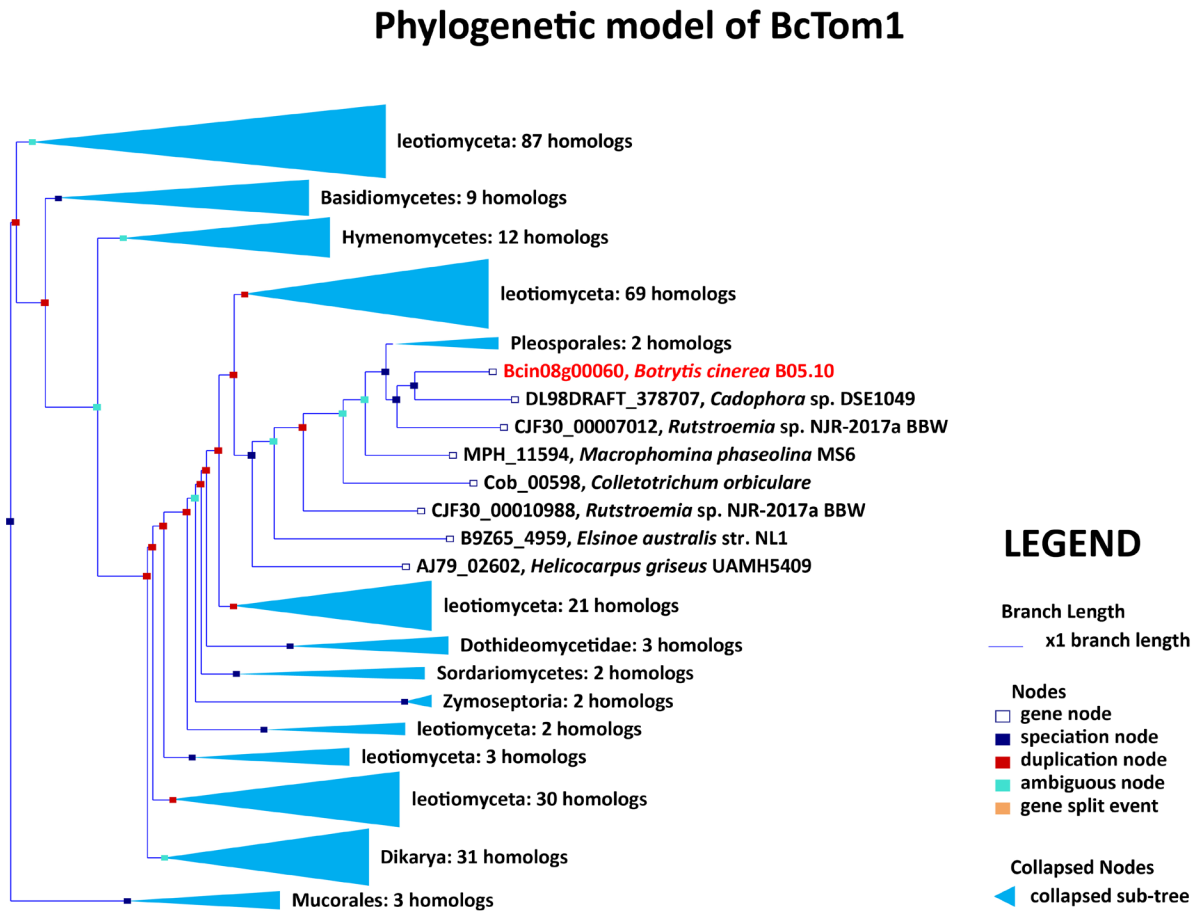
Supplementary data

Supplementary Table S1. Primers used in this chapter for RT-qPCR analysis of α-tomatine-responsive genes in *B. cinerea*.

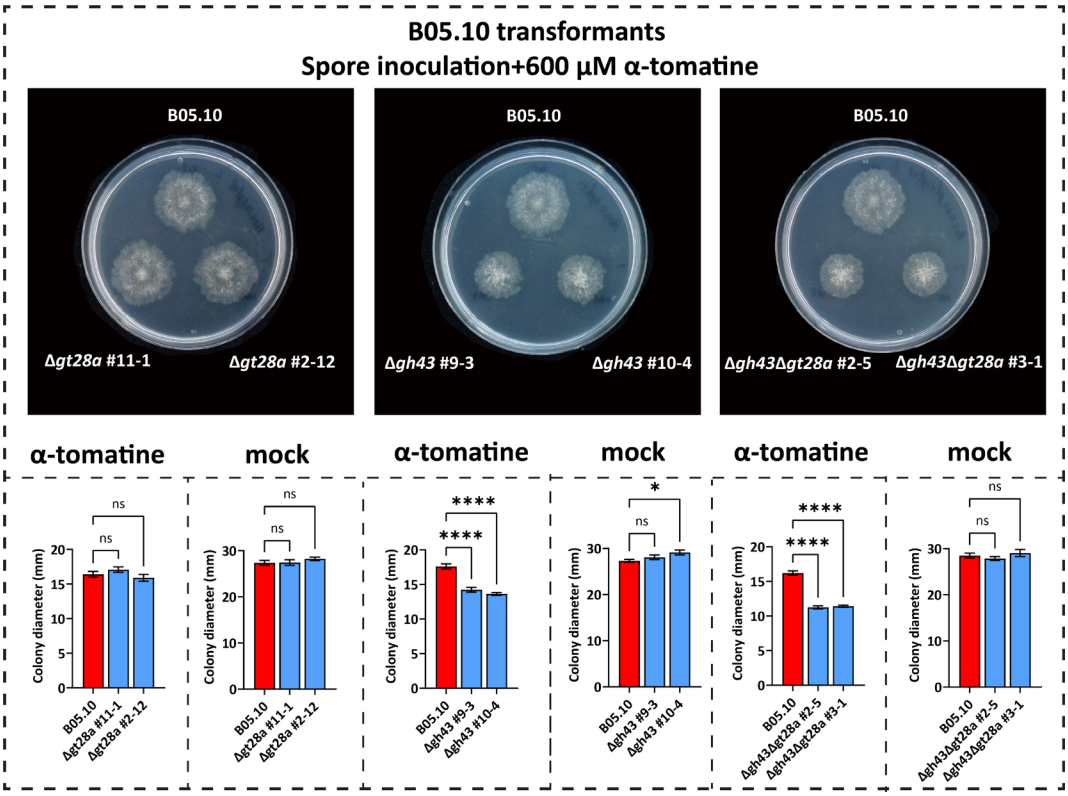
Primer names	Sequence (5'->3')
Bcin09g00800_RTA1a_F	TCCGCAAGAAGTGGATGACC
Bcin09g00800_RTA1a_R	GCCAAAGCGCTAGTTGTTCC
Bcin06g00470_RTA1b_F	CAGCATGAGTTCACTTGGCG
Bcin06g00470_RTA1b_R	GAGCATGAGCAATTGGTGCC
Bcin05g02090_RTA1c_F	CACCGACGGCGAAAAACATT
Bcin05g02090_RTA1c_R	CAACTTGGACGACGAGACCA
Bcin02g09400_RTA1d_F	ATTCGTTGTCGGAGACGTCC
Bcin02g09400_RTA1d_R	GACGGCGACGACAATAAAGC
Bcin08g00060_GH43_F	CGCTGACTTGTTTCACGACG
Bcin08g00060_GH43_R	CTCCATCCGCTTTGGACTCC
Bcin08g00070_GT28a_F	GCAGCTTGTGAGGTCGTCTA
Bcin08g00070_GT28a_R	GGCTTCTCCCAATCGAGGTC
Bcin09g02710_GT28b_F	TCTCCTTTCAGAAGCCGCAT
Bcin09g02710_GT28b_R	GTGCAGAACCGCGTCATAAG
Bcin01g07660_ABC_1F	ATTGCAAGTGGGCATTTCGG
Bcin01g07665_ABC_1R	TGGTATGACGGTTCCATAAGC
Bcin01g07660_ABC_2F	GGGAGCATCTGGATCTGGC
Bcin01g07665_ABC_2R	TTTCTCGTCGTACTCCTCGG
Bcin11g03430_SMT3_F	TCAAATCTACTCCTGCATTCATCG
Bcin11g03430_SMT3_R	GTTCAAGTGCTCGGAAACAGG
Bcin01g08040_TUBA_F	ACCGGCCAATGTGGTAACC
Bcin01g08040_TUBA_R	TGGTACCTGGCTCCAAATCG



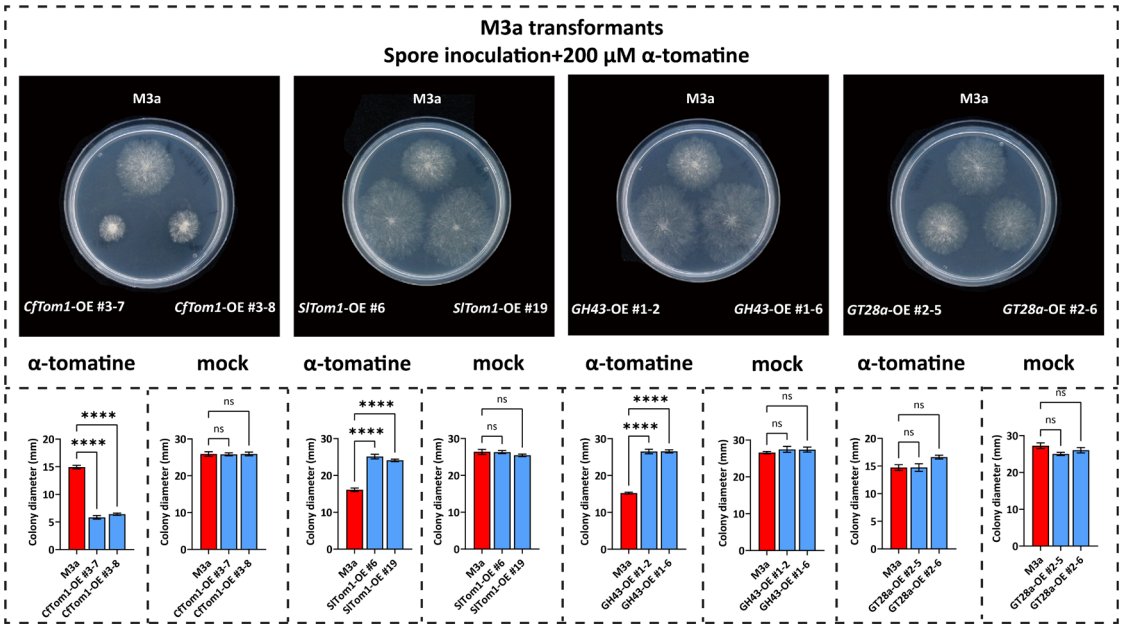
Supplementary Figure S1. Relative expression of α-tomatine-responsive genes in B05.10 after mock treatment.



Supplementary Figure S2. Phylogenetic model of BcTom1



Supplementary Figure S3. Sensitivity test of B05.10 transformants to α-tomatine. Fungal colony growth were from 2 µL droplet of spore suspension (1000 spores/ µL) inoculated on GB5-10 mM sucrose-10 mM phosphate plates containing 600 µM α-tomatine. Colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01; **** p-value <0.0001). ns indicates no significant difference.



Supplementary Figure S4. Sensitivity test of M3a transformants to α-tomatine. Fungal colony growth were from 2 µL droplet of spore suspension (1000 spores/ µL) inoculated on GB5-10 mM sucrose-10 mM phosphate plates containing 200 µM α-tomatine. Colony diameters were measured at 3 dpi. The asterisks represent statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value < 0.01; **** p-value <0.0001). ns indicates no significant difference.

Chapter 6

The role of two *GLYCOALKALOID METABOLISM* genes in α-tomatine biosynthesis and basal defense in tomato

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Abstract

Steroidal glycoalkaloids (SGAs) are important plant defense compounds with antimicrobial activities. α-Tomatine as the major SGA in tomato has received great attention regarding its putative role in plant basal defense. The biosynthetic pathway of SGAs including α-tomatine has been reported and involves multiple clustered genes designated as *glycoalkaloid metabolism* (*GAME*) genes. In this chapter, we generated single knockout (KO) mutants of *GAME4* and *GAME2* via a CRISPR/Cas9-based genome editing strategy. The deletion of *GAME4* abolished α-tomatine accumulation, whereas *GAME2*-KO mutants still accumulated large amounts of α-tomatine similar to the wild type indicating that the *GAME2* gene, although proclaimed to catalyze the transfer of UDP-xylose to β_1-tomatine, is not essential for α-tomatine biosynthesis. Infection assays with the fungus *Botrytis cinerea* and the oomycete *Phytophthora infestans* indicated that *GAME4*-KO mutants were more susceptible to these pathogens. Up-regulation of α-tomatine-responsive genes in *B. cinerea* was still observed during infection on α-tomatine-deficient *GAME4*-KO plants, suggesting that these plants produce other secondary metabolites that can induce the fungal transcriptional response to α-tomatine. The mode of action of α-tomatine and its contribution to basal defense of tomato against pathogens and pests is discussed.

Introduction

Tomato (*Solanum lycopersicum*) from the Solanaceae (nightshade) family is an economically important vegetable crop and serves as a rich source of nutrition worldwide. However, the sustainable yield of tomato is threatened by pathogenic fungi such as grey mould (*Botrytis cinerea*), leaf mould (*Cladosporium fulvum*), Verticillium wilt (*Verticillium dahliae*), by pathogenic oomycetes such as late blight (*Phytophthora infestans*), and by viruses such as Tomato yellow leaf curl virus as well as by herbivorous insects (Arie et al., 2007; Blancard, 2012; Panthee and Chen, 2010; Nowicki et al., 2012). This highlights the necessity of exploring endogenous resistance traits to facilitate green and sustainable agriculture. One option is to exploit the reservoir of endogenous tomato antimicrobial secondary metabolites with defensive roles (Bednarek et al., 2012).

Steroidal glycoalkaloids (SGA) are a subgroup of saponins constitutively present in *Solanaceae* and *Liliaceae* families (Cárdenas et al., 2015). They not only possess antinutritional effect (bitterness) but are also considered as phytoanticipins protecting the plants from attack by pathogens and herbivores due to their high concentration and broad-spectrum antimicrobial as well as insecticidal activities (Sandrock and VanEtten, 1998; Sun et al., 2021; Zhao et al., 2021). In tomato, α-tomatine is the major SGA and it accumulates in vegetative tissues and green fruits at concentrations exceeding 1 mM (Kozukue et al., 2004; You and van Kan, 2021).

The biosynthetic pathway of SGAs starts from the precursor cholesterol. Through a series of steps mediated by the enzymes encoded by *GLYCOALKALOID METABOLISM* (*GAME*) genes including hydroxylation (*GAME7, GAME8*) and E-ring closure (*GAME11* and *GAME6*), cholesterol is converted into a furostanol-type saponin (Itkin et al., 2013). GAME4, a cytochrome P450 protein, catalyzes the first dedicated step from the furostanol-type aglycon towards SGAs. GAME4 is considered to play a central role in SGA biosynthesis. Silencing of *GAME4* in tomato resulted in overaccumulation of intermediates such as phytosterols (Itkin et al., 2013). It is noteworthy that the SGA intermediate tomatidine, the aglycon of α-tomatine, is toxic to tomato cells. This was demonstrated by the necrosis observed upon infiltration of commercial tomatidine (Ökmen et al., 2013) and by the morphological defect in transgenic *GAME1*-silenced tomato plants accumulating higher amounts of tomatidine (Itkin et al., 2011). In tomato, the phytotoxic effect of tomatidine can be relieved by four consecutive glycosylation steps (catalyzed by GAME1, GAME17, GAME18 and GAME2) which lead to the production of α-tomatine (Itkin et al., 2013). α-Tomatine is then stored in the vacuole until fruit ripening, and is subsequently stepwise converted into less bitter and less toxic SGAs such as esculeoside A (Cárdenas et al., 2015). This conversion process also

requires a transporter protein in the tonoplast of tomato cells that relocates α-tomatine from the vacuole to the cytosol to make α-tomatine accessible to cytosolic enzymes (Kazachkova et al., 2021). The indispensable role of this intracellular translocation process in α-tomatine metabolism was demonstrated by the high α-tomatine content in mature fruits of the transporter KO mutants (Kazachkova et al., 2021).

The toxicity of α-tomatine to fungi is generally attributed to the disruption of fungal plasma membranes which requires the complexing with 3β-hydroxy sterol (Steel and Drysdale, 1988; You and van Kan, 2021). *Phytophthora* spp. which lack the ability to synthesize sterols exhibited relatively strong *in vitro* resistance to α-tomatine, but became more sensitive with the incorporation of exogenous sterols into the membrane from the culture medium (Steel and Drysdale, 1988). Moreover, α-tomatine can cause electrolyte leakage in a variety of plants with the exception of nightshade plants tomato and potato, which is probably related to the high proportion of glycosylated sterols in the latter plants (Duperon et al, 1984; Steel and Drysdale, 1988). In addition to the modification of membrane sterols, emerging evidence also highlights the importance of vacuolar localization to avoid self-toxicity of SGAs. The overexpression of the tonoplast transporter in tomato led to hyperaccumulation of α-tomatine in the cytosol and resulted in phytotoxicity (Kazachkova et al., 2021). Besides, α-tomatine can also inhibit the growth of bacteria which do not have membrane sterols (Enya et al., 2007). Moreover, α-tomatine secreted from tomato roots was reported to manipulate the rhizosphere microbiome (Nakayasu et al., 2021).

Detoxification of α-tomatine has been reported in many tomato pathogens. Degradation of α-tomatine into less toxic breakdown products is the predominant strategy which employs secreted glycosyl hydrolases (GH) referred to as “tomatinase”. So far, a complete picture of tomatinase activities has been uncovered of three types of catalytic activities attributed to three distinct GH families (GH10, GH3 and GH43) (You and van Kan, 2021; **Chapter V**). Besides, non-degradative mechanisms for tolerance to α-tomatine have also been unveiled in Chapter IV which probably include membrane modification.

The constitutive presence of antimicrobial secondary metabolites can play an important role in basal defense (Osbourn et al., 1996; Zaynab et al., 2021). Saponin-deficient oat plants exhibited compromised resistance to fungal diseases (Papadopoulou et al., 1999). The fungal pathogen *Nectria haematococca* could only infect the mature tomato fruit (low in α-tomatine) but not the green fruit which is abundant in α-tomatine (Sandrock and vanEtten, 2001). A recent study from our group has shown that the *B. cinerea* grape isolate M3a is very sensitive to α-tomatine and could barely colonize tomato leaves (Chapter IV). However, the definitive role in innate immunity of SGAs in general, and the tomato SGA α-tomatine in particular, has not been addressed using transgenic tomato with altered levels of α-tomatine. In this study, we generated tomato single KO mutants of *GAME4* and *GAME2* via CRISPR/Ca9 as well as overexpression

lines of each gene. We describe the effects of the deletion and overexpression of the *GAME4* and *GAME2* genes on tomatine levels and susceptibility to various microbial pathogens.

Results

CRISPR/Cas9-mediated mutagenesis of *GAME4* and *GAME2* genes in tomato

In order to generate α-tomatine-deficient tomato plants, a CRISPR/Cas9 genome editing strategy was employed to generate single KO mutants of *GAME2* and *GAME4* with four sgRNAs targeting the open reading frame (ORF) of each gene, in the background of *S. lycopersicum* cv. Moneymaker (MM). A high incidence of biallelic mutations was observed in the T0 generation of primary transformants: six out of seven *GAME2*-KO lines and all the six *GAME4*-KO lines tested carried mutations in both alleles of the target genes. Deletions ranging from 1 bp to 1370 bp were obtained in the ORFs of both *GAME2* and *GAME4* in the T0 generation and were inherited by the T1 plants. **Figure 1** illustrates the positions and sizes of deletions in each gene from four independent homozygous mutants that were obtained in the T1 generation and were analyzed in more detail. In most cases, the deletions were very near to the sequences targeted by the sgRNA. However, the *GAME4*-KO line #6-7 has a 617 bp deletion which starts 226 bp downstream the predicted cleavage site of sgRNA1 and ends 229 bp downstream the predicted cleavage site of sgRNA2.

Screening for putative homozygous overexpression lines of *GAME2* and *GAME4*

Overexpression transformants of *GAME2* and *GAME4* were generated aiming to increase the α-tomatine contents in tomato. Genomic DNA of T1 plants was used to screen for putative homozygous transformants by reverse-transcription quantitative PCR (RT-qPCR) using the tomato housekeeping gene *GAPDH* as a reference (**Supplementary Figure S1**). Transgenic lines with double the copy numbers as compared with its T0 parental line were kept for seed production.

Analysis of tomatidine and α-tomatine contents in tissues of KO plants

Relative concentrations of α-tomatine and tomatidine were analyzed by liquid chromatography triple quadrupole tandem mass spectrometry (LC-QqQ-MS) in four different types of tissues including young leaves, mature leaves, stems and roots from *GAME2*-KO, *GAME4*-KO and wild-type (WT) MM plants. The accumulation of α-tomatine and tomatidine was abolished in *GAME4*-KO with only trace amounts of α-tomatine and tomatidine detected (**Figure 2**), possibly due to cross-contamination from the previous runs in LC-QqQ-MS. Strikingly, all the four independent *GAME2*-KO lines produced α-tomatine at a similar concentration as the WT MM (**Figure 2**). Among the four tissues sampled, the leaves contained by far the

highest concentration of α-tomatine but not of tomatidine. The roots contained around 10 times less α-tomatine than young leaves but accumulated the highest content of tomatidine as compared to other tissues, with almost equal levels of α-tomatine and tomatidine (**Figure 2**). Finally, stems had the lowest concentration of both α-tomatine and tomatidine.

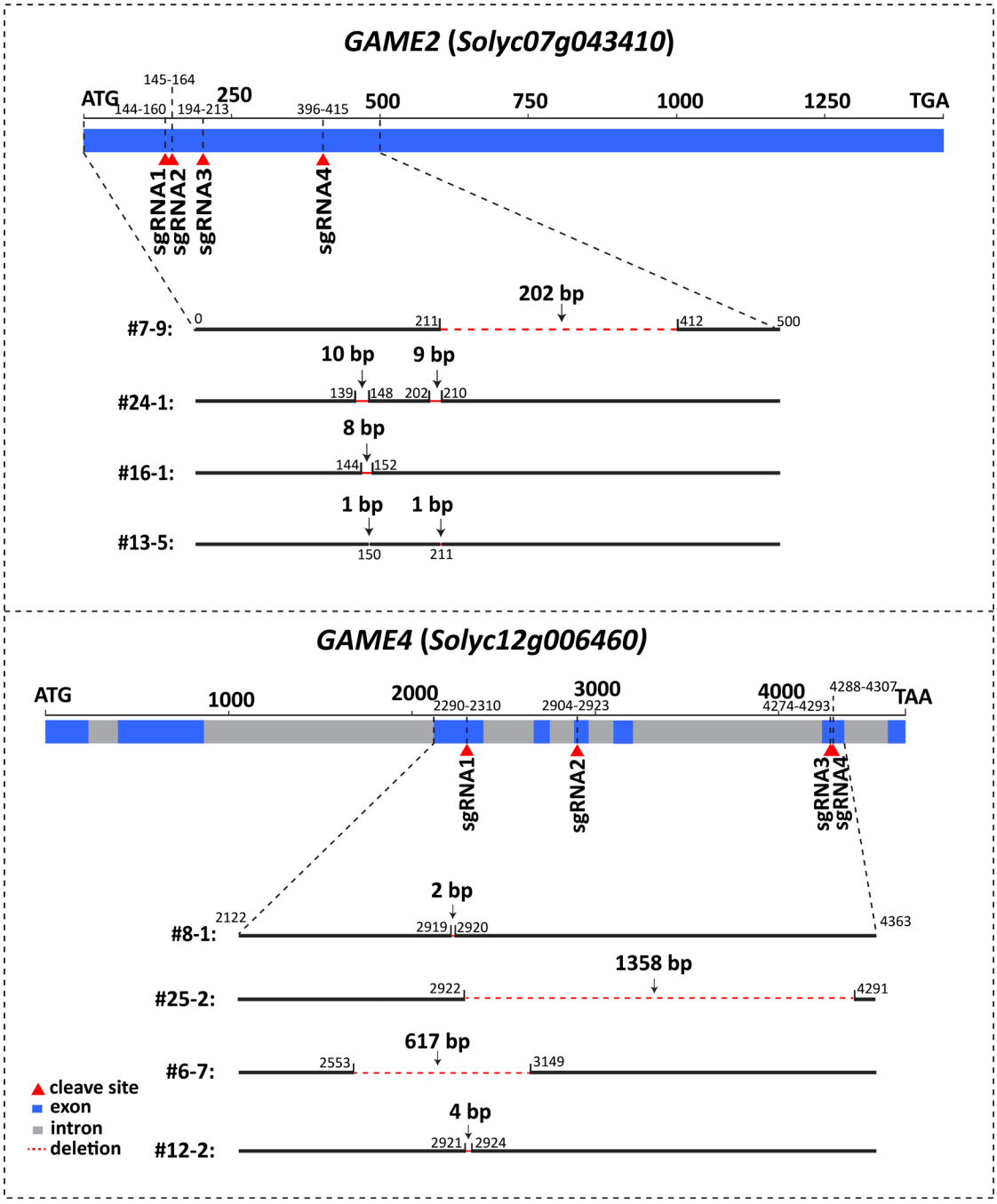

Figure 1. Scheme of CRISPR/Cas9-mediated KO of *GAME2* and *GAME4*. Positions of sgRNAs and their predicted cleavage sites are indicated by coordinates and red arrows, respectively. Deletions are illustrated by red dotted lines, the coordinates of the deleted nucleotides are provided, starting counting from the start codon.

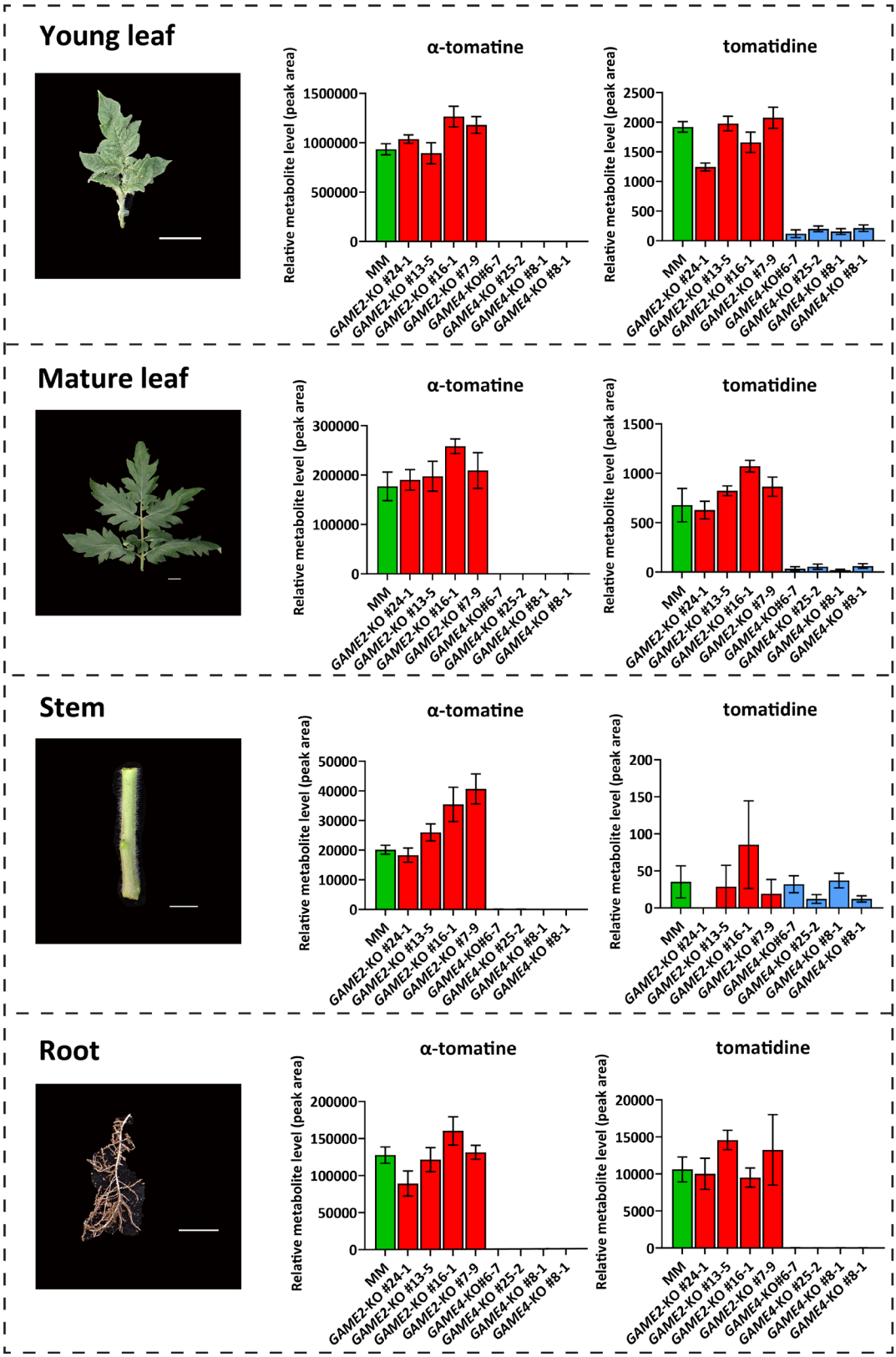

Figure 2. Content of α-tomatine and tomatidine in different tissues of *GAME2*-KO, *GAME4*-KO and MM. The numbers underneath the columns indicate the plant genotypes tested. Error bars are standard error of mean (SEM) of five biological replates. Scale bar indicates 2 cm.

Expression profile of α-tomatine-responsive genes during infection on *GAME4*-KO

The transcriptional up-regulation of multiple α-tomatine-responsive genes was first revealed during *in vitro* growth of *B. cinerea* in presence of α-tomatine (**Chapter V**). *BcTom1* and *BcGT28a* genes played important roles in tomato infection and their expression was induced upon inoculation on tomato leaves but not on *Nicotiana benthamiana* or *Phaseolus vulgaris* which do not accumulate α-tomatine. Therefore, we analyzed the transcription of several α-tomatine-responsive genes during infection on *GAME4*-KO leaves by RT-qPCR. Unexpectedly, up-regulation of these genes was observed from 24 hpi onwards, which coincides with the induction of plant cell death and the development of primary lesions (**Figure 3**).

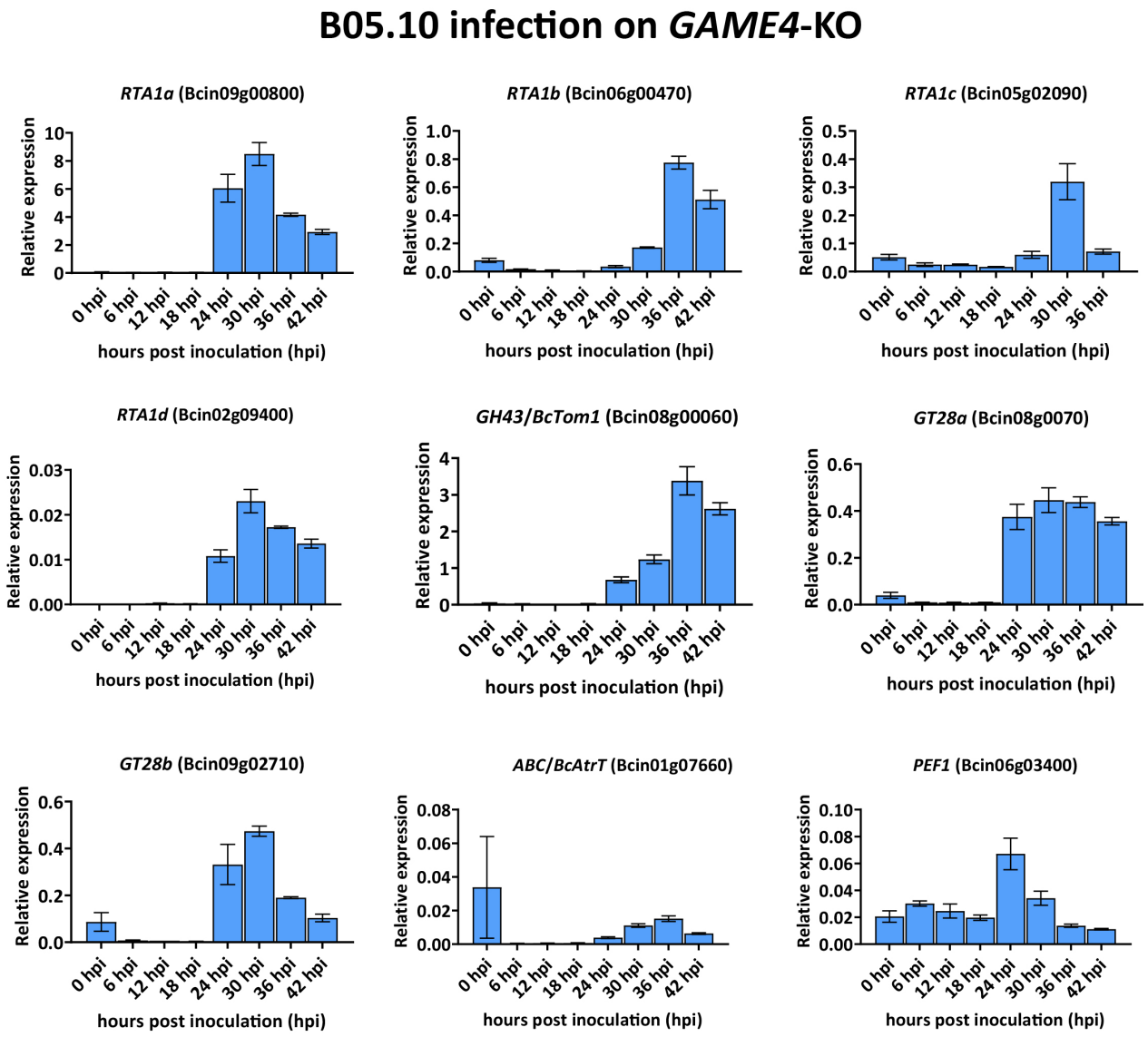

Figure 3. Relative expression of α-tomatine-responsive genes in *B. cinerea* during infection on the leaves of *GAME4*-KO #6-7. Error bars are standard error of mean (SEM) of three biological replates.

Leaf infection assays of *B. cinerea* and *P. infestans* on *GAME2*-KO and *GAME4*-KO

To assess the disease susceptibility in the transgenic plants lacking α-tomatine, leaf inoculations were performed with *B. cinerea* and *P. infestans* on *GAME4*-KO and *GAME2*-KO plants, as well as on the non-transgenic MM which produces wild type levels of α-tomatine. Significantly larger lesions of *B. cinerea* and *P. infestans* were observed on *GAME4*-KO as compared with MM, whereas *GAME2*-KO did not display increased susceptibility (**Figure 4**).

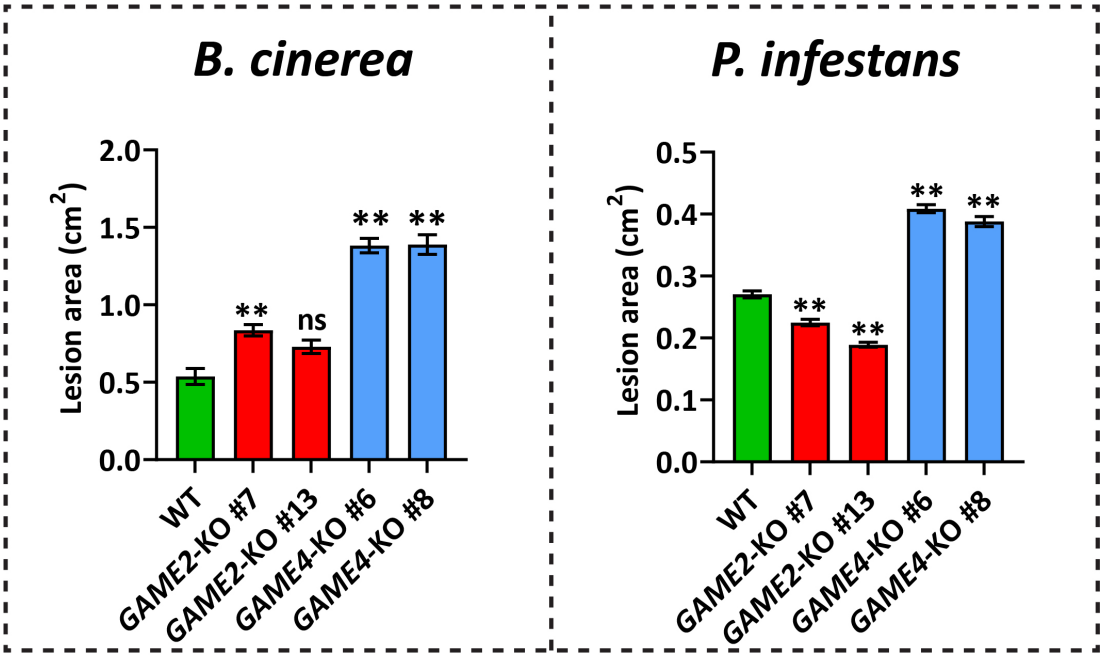

Figure 4. Lesion sizes of *B. cinerea* and *P. infestans* on leaves of *GAME2*-KO, *GAME4*-KO and MM at 3 days post inoculation (dpi). Area of lesions was measured by imageJ. Error bars are standard error of mean (SEM). The asterisks indicate statistically significant differences between KO plants and WT plants determined by student's t-test (** p-value <0.01). ns indicates no significant difference.

***P. infestans* exhibits sterol-independent *in vitro* sensitivity to α-tomatine**

To study the sensitivity of *P. infestans* to α-tomatine, a plate assay was performed with different concentrations of α-tomatine in the presence and absence of sterols. The growth was monitored over a period of two weeks and colony diameters were measured at regular intervals to quantify growth (**Figure 5**). *P. infestans* radial growth on α-tomatine plates was slower than the control plates both in the absence and the presence of sterols in the medium (**Figure 5**). The inhibitory effect was more pronounced at higher α-tomatine concentration. Moreover, addition of exogenous sterols in the medium significantly promoted the radial growth of *P. infestans* but simultaneously increased its sensitivity to α-tomatine (**Figure 5**).

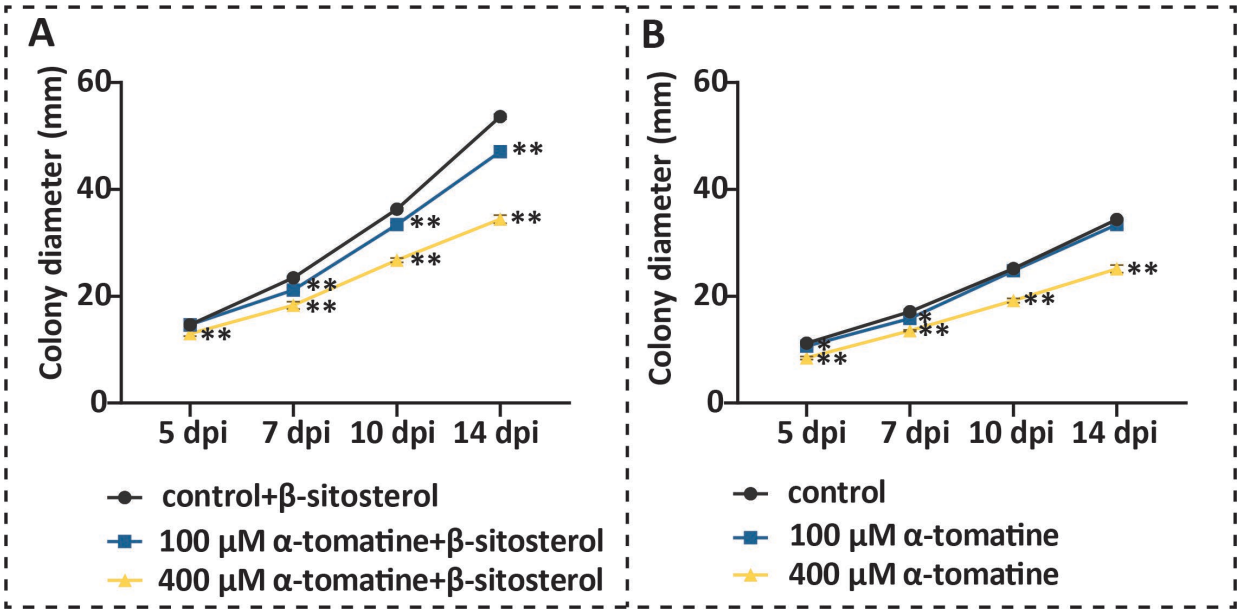

Figure 5. *In vitro* growth of *P. infestans* under different conditions over 14 days. Growth was measured as colony diameter at 5 dpi, 7dpi, 10 dpi and 14 dpi in the presence of 50 µM β-sitosterol (A) or absence of sterols (B). Error bars are standard error of mean (SEM) of four biological replates. The asterisks indicate statistically significant differences determined by the student's t-test (* p-value <0.05; ** p-value <0.01).

Discussion

In order to study the contribution of α-tomatine to basal resistance of tomato to microbial pathogens, we generated knockout and overexpression lines in two different genes, *GAME4* and *GAME2*, which were reported by Itkin et al. (2013) to catalyze the first and last dedicated steps of the α-tomatine biosynthetic pathway. Four independent homozygous *GAME4*-KO lines indeed lost the ability to produce α-tomatine, as well as tomatidine. Surprisingly, all four independent homozygous *GAME2*-KO lines produced normal levels of α-tomatine. The deletions in the coding sequence of *GAME2* were close to the start codon and all caused a frameshift that should abolish the production of the *GAME2* protein in the homozygous mutant lines. The observation that *GAME2*-KO plants accumulated normal levels of α-tomatine demonstrates that GAME2 is not essential for α-tomatine biosynthesis in tomato. Itkin et al. (2013) has shown that *GAME2* clusters in the tomato genome on chromosome 7 with three other glycosyltransferase genes (*GAME1*, *GAME17*, *GAME18*) that are reported to be responsible for the first three steps in the glycosylation cascade of tomatidine. The tomato GAME1/17/18/2 cluster is highly syntenic with the potato SGA biosynthetic cluster (Itkin et al., 2013). The protein sequence of GAME2 is orthologous to potato SGT3, which catalyzes the transfer of UDP-rhamnose (a hexose) to generate the potato SGAs α-solanine and α-chaconine (Itkin et al., 2013). On the contrary, GAME2 is supposed to use UDP-xylose (a pentose) as the donor-substrate.

Evidence of the existence of β_1-tomatine rhamnoside in tomato is lacking, despite numerous studies that have analyzed the profiles of SGAs in tomato and potato. The fact that *GAME2*-KO plants still produce α-tomatine and that GAME2 is homologous to a potato rhamnosyltransferase thus questions the functional annotation of the GAME2 gene product proposed by Itkin et al. (2013). The tomato genome contains multiple glycosyl transferase genes, but none of these shows significant levels (>50%) of protein sequence identity to GAME2. Furthermore, the transcription of *GAME2* in tomato is restricted to young, developing vegetative tissues. Transcripts of *GAME2* are undetectable in adult, fully expanded leaves, both by RT-qPCR and RNAseq (**Supplementary Figure S2**). By contrast, transcript levels of the other three *GAME* genes that mediate the successive glycosylation of tomatidine, are relatively high in all tissues. We should therefore consider the possibility that GAME2 is not involved in α-tomatine biosynthesis. Instead the final glycosylation of β_1-tomatine might require a xylosyltransferase, encoded by a glycosyl transferase gene distinct from GAME2. Alternatively, the final glycosylation of β_1-tomatine might be mediated by GAME2 and a second xylosyltransferase, unrelated in sequence, having functional redundancy with GAME2. Plant genes encoding glycosyltransferases are from highly diverse gene families, and the same substrate specificity can be observed for glycosyltransferases with low homology (Schuman et al., 2007). One suitable approach to discover the β_1-tomatine UDP-xylose transferase in tomato could be to adopt an activity-directed protein purification strategy as described for the identification of the phloretin glycosyltransferase gene in apple (Wang et al., 2020).

The occurrence of up-regulation of α-tomatine-responsive *B. cinerea* genes even during infection on *GAME4*-KO leaves which do not have α-tomatine suggests the presence of other inducers. The silencing of *GAME4* in tomato has been shown to lead to increased accumulation of non-nitrogenous steroidal saponins including uttroside B (Itkin et al., 2013). In **Chapter V** we have shown that the expression of α-tomatine-responsive genes can also be induced by the steroidal saponin digitonin from *Digitalis purpurea*. Thus, it is conceivable that enhanced synthesis of steroidal saponins such as uttroside B caused by *GAME4* deletion can induce the expression of α-tomatine-responsive genes in *B. cinerea* during infection. However, the steroidal saponin content in *GAME4*-KO plants remains to be verified. If indeed certain steroidal saponins hyper-accumulate in *GAME4*-KO plants, they might not provide similar level of antifungal activity as α-tomatine and therefore increased susceptibility to *B. cinerea* and *P. infestans* can be still observed in *GAME4*-KO plants.

Besides having an effect on basal resistance to pathogens in above-ground tissues, the secretion of α-tomatine from roots was reported to influence the tomato rhizosphere microbiome and could potentially affect the plant fitness indirectly when *game4* mutant plants are growing in non-sterile soil (Trivedi et al.,

2020; Nakayasu et al., 2021). The effect of α-tomatine and its biosynthetic intermediates on the rhizosphere microbiome can be further explored by generating α-tomatine-deficient plants blocked at different steps of biosynthesis, and studying the influence of the accumulation of different glycosylated intermediates on bacterial communities.

In this study, we observed that the roots of MM displayed a very distinct ratio of α-tomatine and tomatidine, as compared to leaf and stem samples (**Figure 2**). While the α-tomatine : tomatidine ratio in leaves was approximately 500 : 1, it was only 10 : 1 in the roots (**Figure 2**). However, it was reported that accumulation of α-tomatine in roots differs between genotypes (Kozukue et al., 2004) and further studies are required to establish how strongly the α-tomatine : tomatidine ratios and contents differ between tomato genotypes. The accumulation of α-tomatine in the roots and its secretion into the rhizosphere is relevant because of its possible impact on the microbial community in the rhizosphere (Nakayasu et al., 2021). While the α-tomatine concentration in the roots is around 10% of that in young leaves, the tomatidine concentration in roots is 10-fold higher than in young leaves (**Figure 2**). This particular high tomatidine : α-tomatine ratio might be due to the low expression of *GAME1* in roots or to *in planta* degradation of α-tomatine into tomatidine. As tomatidine is reported to be phytotoxic in tomato leaves (Ökmen et al., 2013), further research could explain how root cells withstand such a high level of a toxic metabolite and elucidate the biological role of high concentrations of tomatidine in roots. Besides, in light of the toxicity of tomatidine to plant tissues, its role in defense against parasitic plants colonizing the root can also be investigated.

The main mechanism underlying the toxicity of α-tomatine relies on its sterol binding properties, which cause loss of microbial membrane integrity. As *Phytophthora* spp. and other oomycetes entirely lack sterol biosynthesis, they were shown to display high tolerance to α-tomatine and the sensitivity increased upon incorporation of sterols from the environment (Steel and Drysdale, 1988). However, the plate assay performed in this study clearly demonstrated the inhibitory effect of α-tomatine on *P. infestans* when growing on sterol-free media, although supplementation of sterols in the media indeed increased the α-tomatine sensitivity in *P. infestans*. This observation substantiates the important contribution of sterols to α-tomatine sensitivity. However, the toxicity of α-tomatine to *P. infestans* in the absence of sterols indicates that α-tomatine also exhibits a sterol-binding independent toxic action, which can also be inferred from the reports of growth inhibition of bacteria by α-tomatine (Enya et al., 2007). *In vitro* studies using artificial vesicles have demonstrated the essential role of the presence of membrane sterols in membrane leakage caused by α-tomatine (Keukens et al., 1992; Roddick and Drysdale, 1984; Steel and Drysdale, 1988). Thus the sterol-independent toxicity of α-tomatine might involve modes of action unrelated to membrane

disruption. One of the potential alternative modes of action could be the induction by α-tomatine of programmed cell death as has been reported in the fungus *Fusarium oxysporium* (Ito et al., 2007), however, the requirement of sterols in this process remains to be studied.

Future experiments will focus on further infection assays on α-tomatine deficient (*GAME4*-KO) and overproducing (*GAME4*-OE) transgenic plants with additional microbial pathogens with different tissue specificity or lifestyles than the two pathogens tested thus far, i.e. *B. cinerea* and *P. infestans.* Specifically, these experiments will include the biotrophic tomato leaf mould *C. fulvum*, the vascular wilt fungi *V. dahliae* and *F. oxysporum* f.sp. *lycopersici*, as well as bacterial leaf pathogens such as *Pseudomonas syringae* pv. *tomato.* In addition, experiments with pest insects and nematodes will reveal the breadth of contributions of α-tomatine to the basal defense of tomato plants to attackers. Furthermore, studies on the rhizosphere, phyllosphere and endosphere microbiome in α-tomatine deficient plants may also illustrate the role of α-tomatine in shaping the tomato microbiome.

Materials and methods

Plant and pathogen growth condition

Tomato plants were growing in the greenhouse at Unifarm (Wageningen UR). The compartment was set at 20°C during daytime (16 h) and 19°C at night (8 h) with 60% humidity. 5 to 6-weeks-old tomato plants were used for sampling and inoculation. *B. cinerea* strain B05.10 was used for infection and the growth condition and spore collection were described in **Chapter V**. *P. infestans* strain 88069 was used for leaf infection. Rye sucrose agar (RSA) plates containing 20 µg/mL vancomycin, 100 µg/mL ampicillin and 20 µg/mL amphotericin was used to grow the *P. infestans* colony. The plates were kept in complete darkness at 20°C for 20 days. 10 mL cold sterilized water was added to the growing culture after 11 days to initiate sporogenesis. Then the plates were placed at 4°C for three hours before collecting the zoospores and counting their concentration in a haemocytometer (Optik Labor).

Generation of OE and KO lines in tomato

Full length coding DNA sequences (CDS) of GAME2 and GAME4 were cloned from cDNA of MM and were introduced into binary vector pGWB402 under control of the CaMV35S promoter, using kanamycin for selection in a Gateway cloning strategy. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and used for tomato transformation. The KO lines were generated using a CRISPR/Cas9 genome editing strategy as described in Hanika et al. (2021). In brief, four sgRNAs were designed to target each gene. Transformation was carried out according to Huibers et al. (2013). T0 generations were

screened and the lines carrying mutations in the CDS of target genes were used for seed production. Homozygous mutants were identified in T1 generation and seeds were used for large scale experiments.

Characterization of zygosity of OE lines

Determination of homozygosity of the progenies of T0 OE transformants was carried out in a RT-qPCR based strategy using gDNA as templates according to German et al. (2003). Relative copy numbers of the transgene were normalized to their parental lines, homozygous offspring were used for seed production.

Quantification of α-tomatine and tomatidine

α-Tomatine and tomatidine concentrations were measured in four types of tissues including young leaves, mature leaves, stems and roots with five biological replicates. The frozen samples were freeze dried and 5 mg from each sample was used for extraction. Samples were extracted with methanol containing 0.1% formic acid by 15 min sonication. After centrifugation, supernatant was filtered and used for LC-QqQ-MS.

Plant infection assay

B. cinerea inoculation was performed as described in **Chapter II** and **Chapter V**. In brief, detached mature tomato compound leaves were transferred from greenhouse to the lab and inoculated on the adaxial side of the four main leaflets. *B. cinerea* inoculation was performed with 2 µL droplets containing 2000 spores in GB5-10 mM sucrose-10 mM phosphate medium and incubated in the lab. *P. infestans* inoculation was carried out by pipetting 10 µL of $1.5x10^5$ zoospores/mL water suspension on the leaf surface and incubated in the growth chamber (18°C 16h-8h day/night) with the first 24 h in darkness. Infected leaves were imaged at 3 dpi and the lesion areas were measured using imageJ.

Gene expression analysis

Analysis of expression of α-tomatine-responsive genes identified in **Chapter V** was carried out using RT-qPCR. mRNA was extracted from infected *GAME4*-KO leaves at 0 hpi, 6 hpi, 12 hpi, 18 hpi, 24 hpi, 30 hpi, 36 hpi and 42 hpi using Maxwell™ 16 RNA Purification Kits (Promega). RT-qPCR was performed using a CFX96 Real-time system (Bio-Rad) and SYBR Green Master Mix (Bio-Rad). Primers were listed in **Chapter V**.

α-Tomatine plate assay

The sensitivity of *P. infestans* to α-tomatine was tested by inoculating 5 mm mycelium plugs on plates containing 100 µM and 400 µM α-tomatine in the presence or absence of 50 µM β-sitosterol. α-tomatine was dissolved in methanol containing 0.5% formic acid and β-sitosterol was dissolved in ethanol. Each plate contained a single plug at the center, with 4 replicates per treatment. The plates were sealed and incubated

in the dark at 20°C. The growth of the colonies was monitored and diameters were measured over 2 weeks.

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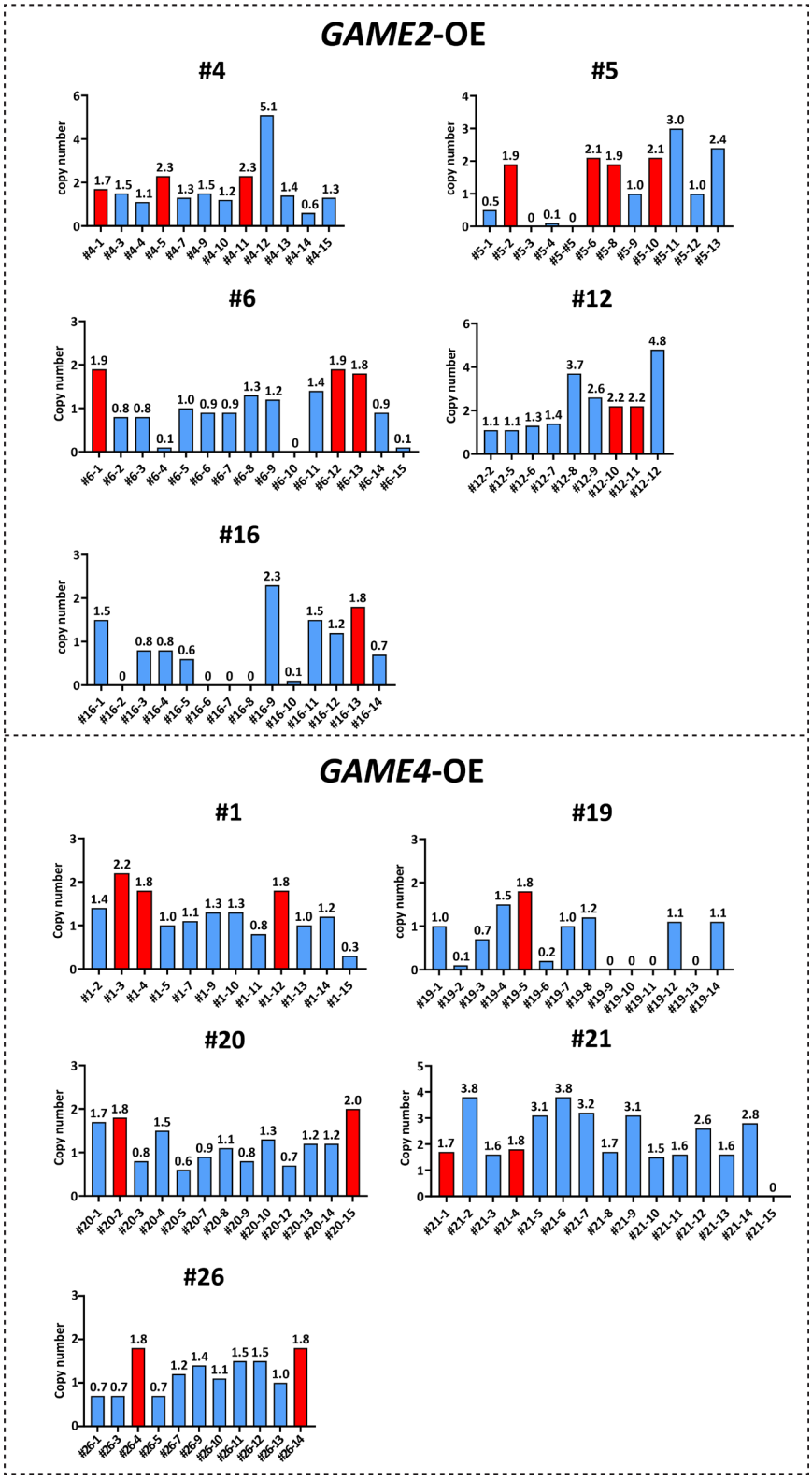
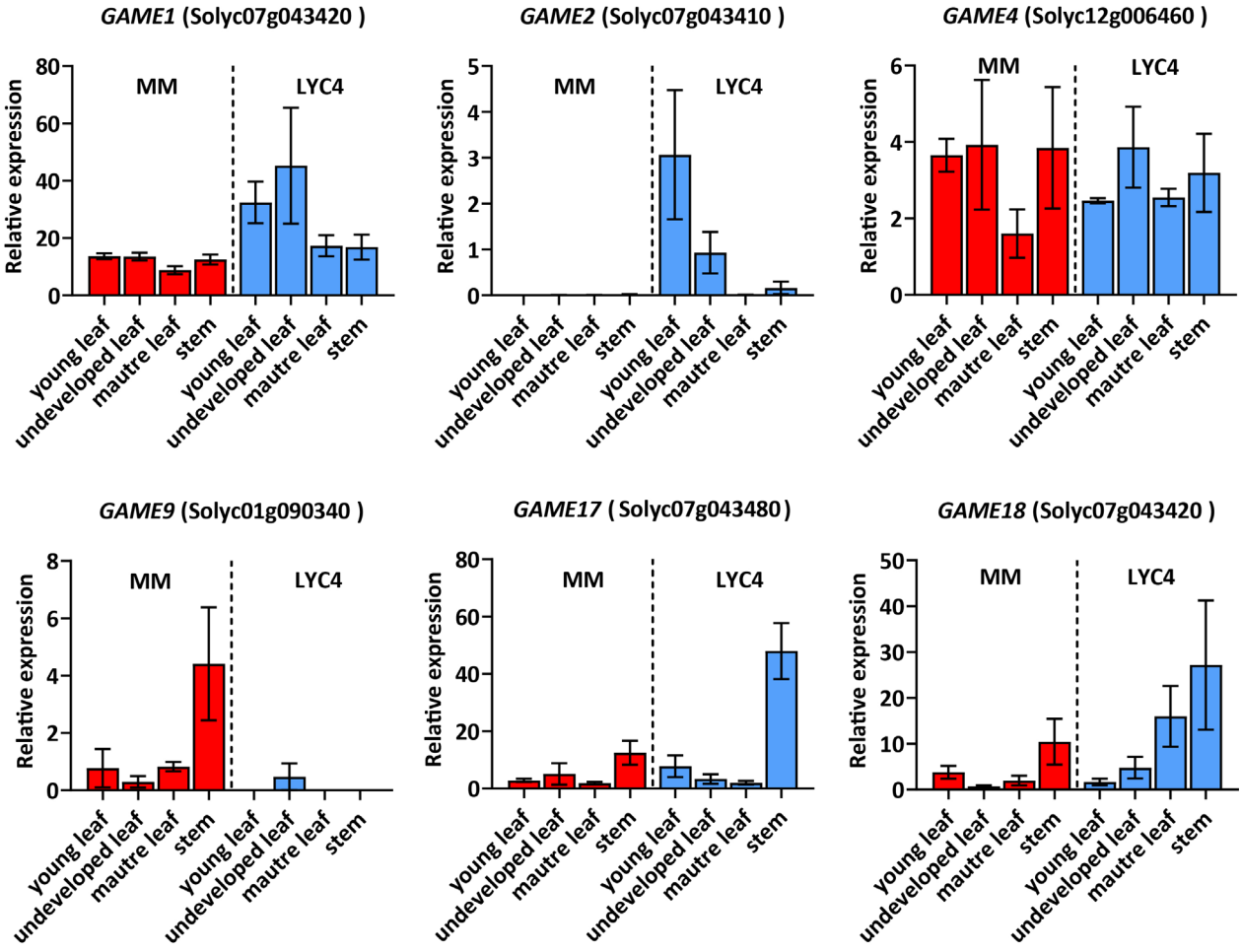
Supplementary data



Supplementary Figure S1. Relative copy number of T1 plants as normalized to its parental line.



Supplementary Figure S2. Relative expression of *GAME* genes in different tissues from *S. lycopersicum* MM and *S. habrochaites* LYC4 analyzed by RT-qPCR. Two house-keeping genes *CAC* and *GAPDH* were used as references. Error bars are standard error of mean (SEM) of three biological replates.

Chapter 7

General discussion

Introduction

Botrytis cinerea, the causal agent of grey mould disease, is capable of infecting more than 1000 species (Elad et al., 2015). It is one of the most well-studied plant pathogens and serves as a model for necrotrophic fungi partially because of the availability of good quality genome information and feasibility of conventional transformation and recently developed CRISPR/Cas mediated genome editing (Leisen et al., 2020; van Kan et al., 2017). The infection strategies of *B. cinerea,* as well as the plant resistance mechanisms have been extensively studied (AbuQamar et al., 2017; Veloso and van Kan, 2018). Based on its scientific and economic importance, *B. cinerea* was ranked in second place among the top 10 fungal pathogens in a survey among scientists active in the field of fungal molecular pathology (Dean et al., 2012). Partial resistance to *B. cinerea* has been reported in the wild tomato species *Solanum habrochaites* and ten quantitative trait loci (QTLs) have been mapped using the introgression line population derived from *S. habrochaites* accession LYC4 (Finkers et al., 2007a; Finkers et al., 2007b). However, all the QTLs only make a minor contribution to resistance to *B. cinerea* which makes it challenging to further elucidate the resistance mechanisms through fine mapping. Besides, *S. habrochaites* is known to produce a large number of bioactive secondary metabolites with antimicrobial and pesticide activity (Jessica et al., 2020; Wang et al., 2020; Zabel et al., 2020). Plant secondary metabolites with antimicrobial activities are potential defense compounds contributing to resistance to plant pathogens. α-Tomatine, a steroidal glycoalkaloid is the major saponin in tomato with well-documented *in vitro* antibiotic activities. Secretion of glycosyl hydrolases that enzymatically degrade α-tomatine into less toxic products is the only detoxification strategy reported in tomato pathogens so far (You and van Kan, 2021). A unique tomatinase activity (β-xylosidase) in *B. cinerea* has been reported more than 20 years ago, however, attempts to clone this gene based on homology to other tomatinase or β-xylosidase genes failed (Quidde et al., 1998; 1999). Besides, although α-tomatine is widely considered as a defense compound, its contribution to resistance against pathogens has not been validated using α-tomatine-deficient plants (You and van Kan, 2021).

Exploiting wild *Solanum* species to improve the resistance to grey mould disease in tomato: still a long way to go

Wild tomato relatives as important germplasm to improve tomato disease resistance

Wild tomato relatives have been successfully used to study resistance to plant pathogens. For instance, Cf genes that originated from wild tomato have been introduced into cultivated tomato to confer resistance to leaf mould disease caused by *Cladosporium fulvum* (Kruijt et al., 2005; van der Beek et al., 1992). Several

resistance genes to tomato yellow leaf curling virus (TYLCV) have been identified in *S. chilense* (Ji et al., 2007; Ji et al., 2008) and *S. habrochaites* (Kalloo and Banerjee 1990; Ji et al. 2009a). Similarly, the studies using wild tomato relatives uncovered several resistance loci and pinpointed the corresponding genes conferring resistance to powdery mildew (Bai et al., 2003; Bai et al., 2008). The cloning and sequencing of a range of resistance genes from cultivated tomato and its wild relatives has given insight into the mechanisms by which they confer resistance to pathogens. Most of the resistance genes characterized in tomato and wild *Solanum* encode intracellular or plasma-membrane bound receptor proteins that recognize ligands (for example, pathogen effectors) and this recognition triggers a hypersensitive (cell death) response that triggers an effective resistance mechanism (van der Burgh and Joosten, 2019). The "gene-for-gene" concept that describes the mode of action of resistance genes is well established for interactions of plants with pathogens that depend on living host plant cells for their reproduction, such as viruses, bacteria and fungi with (facultative or obligate) (hemi-)biotrophic lifestyles (Flor, 1971; Keen, 1990; Loegering and Ellingboe, 198). However, the gene-for-gene concept is not applicable for resistance to necrotrophic pathogens, on the contrary: recognition by plant receptor proteins of effectors from necrotrophic fungi can cause cell death and result in pathogen susceptibility, following an "inverse gene-for-gene" interaction (Friesen et al., 2007; Wolpert et al., 2002). In such situations, resistance to necrotrophs is based on the absence of a plant receptor and is inherited in a recessive manner. The cellular processes and plant genes that contribute to resistance to necrotrophic fungi are poorly understood.

The progress in deciphering resistance mechanisms to *B. cinerea* in wild tomato relatives is limited

Despite the fact that partial resistance to *B. cinerea* in wild tomato species has been reported for at least 20 years (Nicot et al., 2002), there is little progress in identifying any gene(s) contributing to this resistance. So far, the main advance is the mapping of multiple quantitative trait loci (QTL) from several wild species including *S. habrochaites*, *S. lycopersicoides* and *S. neorickii* without further unravelling of the underlying mechanisms (Finkers et al., 2007; Finkers et al., 2008; Davis et al., 2009). This highlights the importance of pyramiding multiple QTLs to obtain a high resistance level but also raises the concern that the combination of QTLs which cover large parts of the wild tomato genomes, might bring undesirable traits to cultivated tomato. Therefore deciphering the genes and mechanisms conferring resistance is crucial to allow a more precise introduction of the resistance traits into the cultivated tomato. However, the low resistance contribution made by each QTL combined with the enormous difference in the transcriptome, even in mock-inoculated plants (Chapter III), make it very challenging to further pinpoint the *S. habrochaites* genes that contribute to resistance through genetic approaches.

Transcriptome analysis to investigate the resistance mechanisms and its challenges

Transcriptome analysis based on RNA-sequencing (RNA-seq) has been widely applied to study host-pathogen interactions. Our transcriptome analysis revealed that, although *S. lycopersicum* and *S. habrochaites* are related and crossable, they differ in the expression of a large number of genes in the absence of *B. cinerea*. For instance, before *B. cinerea* inoculation, there were >8000 differentially expressed genes (approximately equal numbers of up-regulated and down-regulated genes) in the leaf samples of LYC4 compared with MM. Similarly, Chen et al. (2015) reported in total 7505 differentially expressed genes between *S. habrochaites* and *S. lycopersicum*, possibly reflecting the morphological and physiological differences between the species. This high basal transcriptional divergence combined with the widespread transcriptional reprogramming after *B. cinerea* inoculation hampered the interpretation of RNA-seq results from *B. cinerea*-infected *S. lycopersicoides* and *S. lycopersicum* (Smith et al., 2014).

Compatible and incompatible interactions between *B. cinerea* and tomato: stop the unstoppable

A successful *B. cinerea* infection on tomato leaf under controlled lab conditions is manifested as the formation of primary lesions covering the area of the inoculum droplet and subsequent development of massive plant cell death, visible as water-soaked brownish lesions outside the primary lesion. Once the lesion starts to progress beyond the primary inoculation site the fungal infection can hardly be stopped and under favourable conditions will destroy the whole tomato leaf in a few days. However, *B. cinerea* inoculation in tomato leaf sometimes can result in primary lesions that fail to expand. In this situation, the infection is fully contained at the early stage and the fungus is no longer able to colonize the whole tomato leaf. Such non-expanding lesions commonly exhibit a different appearance, visible as black and dispersed necrotic spots (**Figure 1**). The non-expanding lesions are referred to as “incompatible interaction” between *B. cinerea* and tomato, which is an intriguing phenomenon because the appearance of the necrotic spots resembles the symptoms of a hypersensitive response (HR) in plants carrying resistance genes to biotrophic or hemi-biotrophic microbial pathogens, which can also lead to full resistance.

Figure 1. Expanding and non-expanding lesions after *B. cinerea* inoculation on *S. habrochaites* LYC4

Plant basal resistance levels

First of all, the occurrence of incompatible interaction between *B. cinerea* and tomato is affected by the intrinsic (basal) resistance level in the host. It has been reported that *B. cinerea* inoculation on leaves of the wild tomato relative *Solanum lycopersicoides* resulted in a much lower frequency of spreading lesions than on other *Solanum* species including the cultivated tomato *S. lycopersicum* (Guimarães et al., 2004). Besides, the *S. lycopersicum* abscisic acid (ABA) mutant *sitiens* also exhibited a larger percentage of non-expanding lesions than its wild type (Audenaert et al., 2002). In our study, under certain inoculation conditions, *S. habrochaites* accession LYC4 clearly displayed a much higher incidence of non-expanding lesions than *S. lycopersicum* Moneymaker (MM).

Aggressiveness of the strains

In our study, the grape *B. cinerea* isolate M3a deficient in tomatinase activity could barely form expanding lesions on tomato leaves. Under the same inoculation conditions, inoculation with the isolate B05.10 led to a high frequency of expanding lesions, indicating a clear distinction in aggressiveness between isolates. The overexpression (OE) of tomatinase gene in M3a partially restored its virulence on tomato (Chapter V), indicating that the tolerance to α-tomatine makes an important contribution to the virulence of *B. cinerea*. Soltis et al. (2019) quantified the variation in lesion size of 97 *B. cinerea* isolates on domesticated and wild

tomato genotypes and showed that lesion size was significantly affected by large effects of the host and pathogen genotypes. A genome-wide association study provided indications that >1000 *B. cinerea* genes may contribute to quantitative virulence on 7 of the 11 tomato accessions tested in this study, illustrating the complexity of the interaction between *B. cinerea* and tomato (or probably any other host species).

Inoculation conditions

Inoculation conditions deserve more attention in future studies on the *B. cinerea*-tomato interaction. Artificial *B. cinerea* inoculation under lab conditions requires supplementation with nutrients (minerals, sugars and phosphates) in the inoculum to initiate the infection (Benito et al., 1998), whereas inoculation in water does not result in any visible symptoms. The increase of sugar or phosphate concentration, separately, can significantly increase the ability of *B. cinerea* to cause expanding lesions in tomato ABA mutant *sitiens* (Audenaert et al., 2002). In our study, the high resistance in LYC4 (shown as low incidence of expanding lesions when using inoculum composed of 1000 spores/µL, 3g/L GB5 salts, 10 mM sucrose and 10 mM potassium phosphate) could be fully overcome by increasing the sucrose concentration to 50 mM which resulted in a mostly compatible interaction. The spore density in the inoculum also played a role in shaping the fungal infection and the plant responses. The inoculation of the necrotrophic fungal pathogen *Plectosphaerella cucumerina* at high spore density on Arabidopsis induced jasmonic acid (JA)-dependent defenses and massive plant cell death, whereas low spore density inoculation resulted in less plant cell death and unexpectedly led to the activation of salicylic acid (SA)-dependent defenses which is a typical plant response to biotrophic pathogens (Pétriacq et al., 2016). In our study, a counter intuitive observation was that a reduction in *B. cinerea* spore density reverted an incompatible interaction into a compatible interaction in LYC4.

Potential mechanisms underlying incompatible interactions

The molecular basis of incompatible interactions between *B. cinerea* and tomato has not been fully unravelled. The use of a green fluorescent protein (GFP)-tagged B05.10 strain in infection of the wild tomato *S. lycopersicoides* revealed lysis of hyphae and fungal cell death in the non-expanding lesions at 3 dpi, indicating the potential role of plant antifungal metabolites and proteins in inhibiting the fungal progression (Guimarães et al., 2004). By contrast, the restriction of *B. cinerea* to the primary lesion in a tomato ABA mutant was shown to be related to early plant defense responses including ROS production and cell wall fortification (Asselbergh et al., 2007). Furthermore, a model by Veloso and van Kan (2018) proposed that suppression of plant autophagic cell death, especially at early infection stages, is the key to successful fungal colonization. Altogether, the incompatible interaction between *B. cinerea* and its host

plants is an important phenomenon because it resembles the black-and-white outcome that is commonly observed for biotrophic pathogens. The controlled manipulation of the infection outcome (expanding or non-expanding lesion) by altering the inoculation conditions can provide suitable conditions to study the grey mould disease and therefore should receive more attention in future studies.

PACs in basal and induced defense against plant pathogens

Pre-formed PACs

Plants can produce a wide variety of secondary metabolites with antimicrobial activities. Saponins comprising triterpenoids, steroids or steroidal glycoalkaloids are well-studied and play an important role in resistance against phytopathogens (Osbourn et al., 1996). They are considered as typical phytoanticipins because of their preformation in plant cells. However, in some cases the abundance of saponins can be further induced upon pathogen attack, for instance, the concentration of α-tomatine in *Solanum arcanum* increased 10-fold upon inoculation with the fungal pathogen *Alternaria solani* (Shinde et al., 2017).
Studies on avenacins in oat roots illustrated the importance of saponins in plant basal immunity. The wild oat species *Avena longiglumis* lacking avenacin was more susceptible to the fungal pathogen *Gaeumannomyces graminis* var. tritici than the domesticated oat *Avena sativa* (Osbourn et al., 1994). The contribution of avenacin to plant resistance was later validated by the demonstration of compromised disease resistance in oat saponin-deficient (*sad*) mutants (Papadopoulou et al., 1999).

Inducible PACs

The low molecular weight PACs whose synthesis or activation does not occur in healthy tissues but can be induced upon biotic and abiotic stresses are defined as phytoalexins (VanEtten et al., 1994). The indole alkaloid camalexin from the family Cruciferae has broad antimicrobial activities and its role in plant defense has been well-studied in Arabidopsis (Rogers et al., 1996). It can be rapidly produced upon different stimuli including ultraviolet (UV) light irradiation (Nawrath and Métraux, 1999; Mert-Turk et al., 2003; Glawischnig, 2007), silver nitrate treatment (Glawischnig et al., 2004) and pathogen infection (Mert-Turk et al., 2003; Stefanato et al., 2009). Disruption in Arabidopsis of camalexin biosynthetic genes or membrane transporter genes required for its secretion increased its susceptibility to necrotrophic pathogens (He et al., 2019; Nafisi et al., 2007; Stefanato et al., 2009).

Tomato major saponin α-tomatine

In *Solanaceae* such as tomato and potato, several of the biosynthetic genes for the production of steroidal glycoalkaloid saponins, including α-tomatine, α-solanine and α-chaconine are clustered (Itkin et al., 2013). In tomato the synthesis of α-tomatine from the aglycon tomatidine requires four consecutive steps of glycosylation to form the lycotetraose and was proposed to be catalyzed by four independent GTs denominated GAME1, GAME17, GAME18 and GAME2 (Itkin et al., 2013). The phytotoxicity of the aglycon tomatidine was observed in *GAME1*-silenced tomato plants which accumulated high levels of tomatidine and displayed growth retardation and morphological defects (Itkin et al., 2011). Tomatidine has much lower antimicrobial activity than α-tomatine to fungi, including tomato pathogens (Hoagland, 2009). In fact, the other two α-tomatine degradation products β_1-tomatine and β_2-tomatine, only lacking one terminal xylose or glucose, respectively, are also far less toxic to tomato fungal pathogens (Quidde et al., 1998; Sandrock and VanEtten, 1998). Thus, for the tomato plant itself, the four glycosylation steps of the self-toxic tomatidine not only achieve detoxification but are also preciously designed to generate the defense compound α-tomatine. This is mirrored by the detoxification mechanisms reported in multiple tomato pathogens, which employ deglycosylation of α-tomatine albeit differing in the number of sugar residues hydrolyzed (You and van Kan, 2021).

The success of plant pathogens revolves around their tolerance to plant antimicrobial compounds (PACs)

Efflux of PACs

Plant pathogens evolved multiple active mechanisms (with different modes of action) to mitigate the toxicity of the chemically diverse PACs produced by their host plants. One important tolerance mechanism is the efflux of toxic molecules mediated by membrane transporters which can be categorized into two groups: ATP-binding cassette (ABC) transporters and major facilitator family (MFS) transporters (Westrick et al., 2021). These two types of transporters are ubiquitously present in high numbers in the genome of many plant pathogens, especially in pathogens with a broad host range (Coleman and Mylonakis, 2009). For instance, the genome of *B. cinerea* contains more than 40 predicted ABC transporter genes and more than 282 MFS genes (Amselem et al., 2011). The export of PACs from the fungal cytoplasm to the extracellular space can prevent them from acting on their targets such as enzymes essential for viability. Membrane transporters have been reported to not only confer tolerance to plant defense compounds such as camalexin and glucosinolate degradation products but are also involved in resistance against

fungicides (Stefanato et al., 2009; Vela-Corcía et al., 2019).

Enzymatic modification of PACs

Another essential detoxification mechanism is through the modification of PACs to convert them into less toxic compounds. Westrick et al. (2021) summarized the enzymatic modification of PACs by different microbes via a series of reactions including hydrolysis, oxidation, demethylation, methylation, hydroxylation and glycosylation. One well-studied example is the degradation of the tomato saponin α-tomatine through different GHs by tomato pathogens (You and van Kan, 2021). The identification of the gene from GH family 43, designated *BcTom1* and capable of hydrolyzing α-tomatine into the less toxic product, β_1-tomatine, represents a unique tomatinase activity distinct from previously characterized tomatinase activities of other fungal tomato pathogens (**Chapter V**).

The same PAC can be detoxified by different mechanisms

Interestingly, different detoxification mechanisms can collectively act on the same PAC. For instance, the glucosinolate-breakdown products on the one hand can be excreted by the MFS transporter BcmfsG in *B. cinerea* while they can also be hydrolyzed by SsSaxA in *S. sclerotiorum* (Chen et al., 2020). Moreover, *B. cinerea* also contains an ortholog of the *SsSaxA* (Bcin06g00024) and it is located next to the *BcmfsG*. The ABC transporter BcatrB and the extracellular multicopper oxidase BcLCC2 synergistically confer tolerance to 2,4-DAPG in *B. cinerea* (Schouten et al., 2008). The Arabidopsis phytoalexin brassinin can be oxidized, hydrolyzed as well as glycosylated by different plant pathogens (Pedras et al., 2004; Pedras et al., 2007; Pedras et al., 2008).

The impact of PAC conversion products on plant responses

Some enzymatic conversion products of PAC can have an impact on plant defenses and thereby promote pathogen infection. This was illustrated in the tomato pathogen *Septoria lycopersici* which secretes a GH3 tomatinase to convert α-tomatine into β_2-tomatine, which is not only less toxic to microbes but can also suppress plant defense responses. The *S. lycopersici* GH3 is required for the pathogenicity on *Nicotiana benthamiana* through the suppression of plant hypersensitive response by β_2-tomatine (Bouarab et al., 2002). Moreover, the conversion of α-tomatine by fungal enzymes releases tomatidine and lycotetraose, which can either suppress induced defense responses in tomato cells (40 µM tomatidine) (Ito et al., 2004) or induce plant necrosis when infiltrated into tomato (above 100 µM tomatidine) (Ökmen et al., 2013).

Transcriptional activation of genes involved in tolerance against PACs

A characteristic feature of the tolerance mechanisms to PACs is that they are often inducible at the transcriptional level. Expression of genes encoding GH10 tomatinase in *Fusarium oxysporum* f. sp. *lycopersici* and *C. fulvum* can be up-regulated by α-tomatine (Ökmen et al., 2013; Roldán-Arjona et al., 1999). ABC transporter genes such as *BcatrB* can be transcriptionally induced by a wide arrange of compounds including phenolic antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), the phytoalexins camalexin and resveratrol as well as the fungicide fenpiclonil (Schoonbeek et al., 2001; Schoonbeek et al., 2003; Stefanato et al., 2009; Schouten et al., 2008), each of which can be excreted by the BcatrB protein. The expression of *Bclcc*2 can be induced by tannic acid and cupric ions (Buddhika et al., 2020; Schouten et al., 2002). Therefore, we performed RNA-seq analysis aiming to identify *B. cinerea* genes contributing to the tolerance to α-tomatine. This experiment led to the identification of diverse α-tomatine-responsive genes including the tomatinase gene *BcTom1* (**Chapter V**). The expression of α-tomatine-responsive genes could not be induced by the polyene antibiotic nystatin which alike α-tomatine, causes membrane disruption through interaction with fungal membrane sterols. This indicates that the transcriptional response to α-tomatine might be mediated by the direct recognition of the molecule but not depend on the detection of membrane damage. It may also suggest that the interaction with fungal membranes differs between α-tomatine and nystatin. Quidde et al. (1998) reported that the induction of tomatinase activity likely reflected the induced expression of the (then unknown) tomatinase gene in *B. cinerea,* not only in response to α-tomatine but also in response to its biosynthetic intermediates β_1-tomatine, β_2-tomatine or γ-tomatine lacking one or two sugars residues. However, the aglycon tomatidine lacking the entire lycotetraose did not induce the tomatinase activity (Quidde et al., 1998). This observation suggests the essential role of glycosides in the recognition by the fungal cells. Interestingly, we observed that the main α-tomatine-responsive genes can also be induced by a structurally related saponin, digitonin from the foxglove plant *Digitalis purpurea*, which contains five sugar residues and exhibited high toxicity levels.

Tolerance to PACs by modification of its targets

Besides the export and chemical modification of the PACs, our results provided strong indications for additional tolerance mechanisms which do not directly act on the toxic compounds themselves. One strategy to deal with the membrane disrupting activity of PACs is to counteract their toxic effect through membrane repair. This was first reported in the fungus *Neurospora crassa* and involves a penta-EF-hand protein PEF1 (Schumann et al., 2019). The mode of action of PEF1 is conserved among *N. crassa* and *B. cinerea* (**Chapter V**) and requires the recruitment of PEF1 protein to the damaged sites in the membrane.

The relocalization of PEF1 is partially triggered by the influx of extracellular Ca^{2+} resulting from the membrane leakage. Unlike the previously discussed detoxification genes, which are regulated at the transcriptional level, the expression of *Bcpef1* did not significantly change upon treatment with PACs in our study. The plasma membrane repair can serve as a general cellular response to the injury of the membrane and therefore might be involved in the tolerance to different membrane-disrupting toxins. This hypothesis is supported by the observation of recruitment of BcPEF1 to the membrane upon treatment with nystatin, α-tomatine and digitonin. Membrane repair in eukaryotic cells is involved in several processes including exocytosis and it requires additional Ca^{2+} responsive proteins such as annexin (Andrews and Corrotte, 2018). There is probably an important role of these membrane repair pathways in dealing with PACs acting on the fungal membrane.

Exposure of *B. cinerea* to α-tomatine was shown to induce the expression of a series of genes with diverse functions. Besides the tomatinase gene *BcTom1*, genes encoding glycosyltransferases (GT) from the GT28 family and several transmembrane proteins including RTA1-like proteins, as well as an uncharacterized ABC transporter were also up-regulated. This observation supports the hypothesis of the existence of several non-hydrolytic mechanisms for tolerance to α-tomatine. The further characterization of BcGT28a indicated that it can be mobilized to the damaged sites in membranes of *B. cinerea* in response to α-tomatine. We speculate that BcGT28a can modify membrane components such as ergosterol, but experiments to establish its activity thus far remained unsuccessful. The constitutive expression of *BcGT28a* and the resulting putative membrane modification in *B. cinerea* also conferred tolerance to other membrane-disrupting compound digitonin. The induction of *BcGT28a* during tomato infection suggested an important role for this gene in virulence on tomato and its role was confirmed by infection assays using *B. cinerea* knockout (KO) mutants and OE transformants. Altogether, our study has clearly shown that *B. cinerea* actively altered its membrane composition in response to membrane disrupting compounds (**Figure 2**). Different from the tomatinase which specifically mediates the hydrolysis of the lycotetraose and is predominantly present in tomato pathogens, orthologs of BcGT28, BcRTA1 and BcABC genes are ubiquitous among fungi and might participate in tolerance to a broad spectrum of plant compounds disrupting fungal membranes. The discovery of non-hydrolytic mechanisms for tolerance to PACs in our study could possibly be transferred to plant pathogens that infect hosts accumulating membrane-disrupting compounds other than α-tomatine.

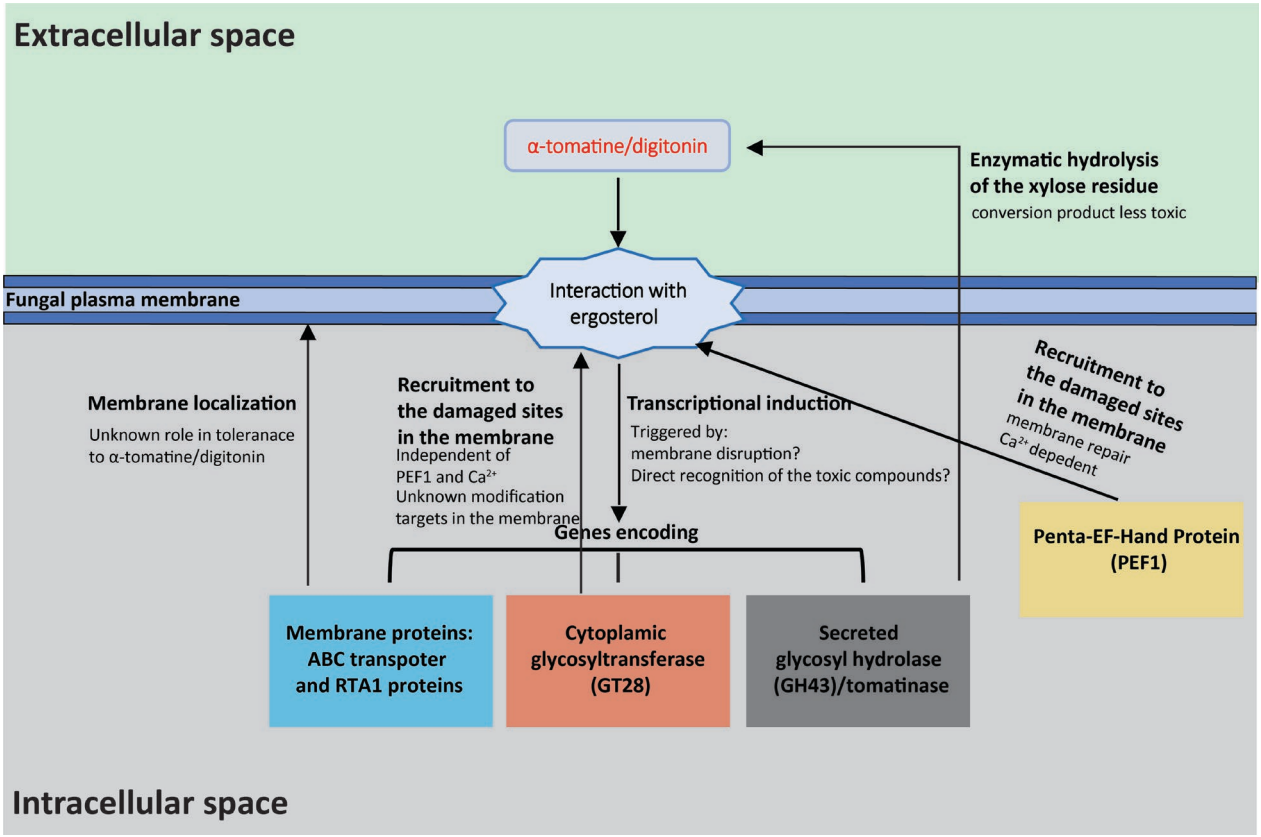


Figure 2. Illustration of fungal response to α-tomatine/digitonin.

Role of tolerance to PACs in the virulence of plant pathogens

Detoxification of PACs can play an important role in the virulence of plant pathogens. *G. graminis* mutants lacking the saponin hydrolyzing enzyme avenacinase became non-pathogenic to avenacin A1-containing oat but could still infect wheat genotypes which do not contain saponins (Bowyer et al., 1995). The export of camalexin by *BcatrB* is required for full virulence of *B. cinerea* on *Arabidopsis* (Stefanato et al., 2009). Another MFS transporter BcmfsG in *B. cinerea* mediating the efflux of glucosinolate-breakdown products is also important for the infection on Arabidopsis (Vela-Corcía et al., 2019). Besides, *BcTom1* KO mutants generated in our study also displayed reduced virulence on tomato. It has in several cases been reported that deletion of PAC detoxification genes did not affect the infection of the pathogen. For instance, the *B. cinerea Bclcc2* KO mutant exhibited similar virulence as the wild type on grape (Schouten et al., 2002). The deletion of GH3 tomatinase in *S. lycopersici* did not affect its virulence on tomato (Martin-Hernandez et al., 2000). However, as mentioned above, plant pathogens can possess multiple detoxification mechanisms even against the same PACs and which might cause functional redundancy. Thereby, the loss of one detoxification function might be compensated by other strategies. Another piece of supporting evidence

is the recently identified triplication of the α-tomatine tolerance genes (*BcTom1* and *BcGT28*) specifically in *B. cinerea* genotypes isolated from tomato, whereas this locus was either present in a single copy or partially/completely absent in genotypes isolated from grapes (Mercier et al., 2021).

Future perspectives

Further perspectives in exploring the resistance mechanisms in wild tomato relatives

Firstly, a high-quality assembly and annotation of *S. habrochaites* LYC4 genome would be of vital importance in deciphering the resistance mechanisms in the future. The currently available assembly (NCBI accession GCA_000577655.1) has ~43,000 contigs and is not annotated. The recent availability of an annotated (partially manually curated) assembly from a different *S. habrochaites* accession may serve as a good starting point (Seong et al., 2022). Annotating the genome of LYC4 might help to define candidate resistance genes that are located within the QTL regions as was done for other wild tomato relatives. For instance, the anchoring of the *S. pennellii* genome assembly to the genetic maps of *S. lycopersicum* identified candidate genes conferring stress tolerance and uncovered the involvement of transposable elements in the evolution of these agronomic traits (Bolger et al., 2014).

Because of the difficulties mentioned above in exploring the resistance mechanisms in wild tomato species, other strategies to understand the interaction of tomato at the *B. cinerea* at the gene level should be considered. Instead of comparing the cultivated tomato with the wild species, transcriptome analysis using the LYC4 introgression lines in the genetic background of *S. lycopersicum* (Finkers et al., 2007) could help to dissect the resistance mechanisms. An alternative strategy might be comparing the transcriptional profiles between different accessions within the same *Solanum* species which exhibit different levels of resistance to *B. cinerea*. *Phytophthora parasitica*-resistant and susceptible accessions of wild tomato *S. pimpinellifolium* were identified and used to analyze the resistance mechanisms to *P. parasitica* using a comparative transcriptome approach (Naveed and Ali, 2018). In addition, the accessions displaying contrasting disease resistance can also be used to generate an intraspecific segregating population which might improve the QTL mapping. This strategy would require more disease assays especially to screen for additional *S. habrochaites* accessions with high level of susceptibility to *B. cinerea*. The occurrence of self-incompatible (SI), self-compatible (SC) and mixed population (MP) mating systems in *S. habrochaites* provides an additional complicating factor for such a strategy (Broz et al., 2017). Furthermore, the observation that the composition of the inoculation buffer and spore density are important determinants of the lesion expansion introduces another experimental variable.

A different option could be to use induced mutagenesis in a resistant, wild tomato species and sequence

the genome of a mutant line with reduced resistance to pinpoint the individual genes conferring susceptibility. A T-DNA insertion mutant population derived from *S. pennellii* has been used to identify genes involved in abiotic stress tolerance (Atarés et al., 2011). However, such a strategy also requires the availability of a well-annotated genome of the wild tomato species.

Overall, there is a need for knowledge on resistance mechanism in wild tomato relatives at the molecular level. The identification of genes and pathways playing positive roles in resistance against grey mould disease is a prerequisite for a more precise marker-assisted breeding to increase resistance in tomato.

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Summary

The broad-host fungal pathogen *B. cinerea* can infect more than 1000 plants and is causing severe economic losses worldwide. However, the current knowledge of the plant-*B. cinerea* interaction, as outlined in **Chapter I,** is insufficient to adequately control the grey mould disease and therefore more research efforts are needed.

So far there are no tomato genotypes reported that display full resistance to *B. cinerea*. Wild tomato relatives such as *S. habrochaites* LYC4 possess partial resistance to *B. cinerea* and are important genetic resources that can be exploited to improve the *B. cinerea* resistance in cultivated tomato, which can only be implemented once we obtain a better understanding of the resistance mechanisms (**Chapter VII**). *B. cinerea* inoculation under lab conditions requires supplementation with nutrients in the inoculum to enable efficient and synchronized infection (Benito et al., 1998). When the inoculum was constituted of 1000 spores/µl suspension in PDB medium, most of the inoculation droplets on both MM and LYC4 leaves formed expanding lesions (compatible interaction). In this situation, the partial resistance in LYC4 was manifested as smaller lesions as compared to MM. We also tested a different type of minimal medium consisting of salts, 10 mM sucrose and 10 mM potassium phosphate. Under this inoculation condition, most of the inoculation droplets in LYC4 formed black dispersed non-expanding lesions (considered to be resulting from an "incompatible interaction"), whereas, the majority of droplets could still form expanding lesions in MM. Remarkably, increasing the sucrose concentration to 50 mM restored the compatible interaction in LYC4 (**Chapter II**). We hypothesized that the nutrient-rich condition was favorable for fungal infection and as a result could mostly overcome the plant defenses in the early stage of infection and lead to a compatible interaction. Inoculation in a low sugar concentration on the contrary reduced the aggressiveness of *B. cinerea*. This rendered the *B. cinerea* infection on the one hand frequently contained in partially resistant LYC4 and on the other hand sufficiently aggressive to break through the resistance in MM and to cause spreading lesions. The controlled, predictable high incidence of incompatible interaction in LYC4 (non-expanding lesions) using the 10 mM sucrose inoculation medium enabled a relatively "black and white" condition to better investigate the resistance mechanisms from LYC4 even if it is conferred by multiple QTLs with minor effects (**Chapter II**).

In **Chapter III**, we performed RNA-seq analysis to investigate the global transcriptional changes in both plant and fungus during *B. cinerea* infection in LYC4 and MM. Importantly, the use of different sucrose concentrations in the inoculum (10 mM vs 50 mM) in LYC4 leaves which led to incompatible and compatible interactions, respectively, were analyzed by RNA-seq. In agreement with previously published

reports of large scale transcriptional reprogramming in plant-*B. cinerea* interactions, we observed that around 20% of the genes in the LYC4 genome were differentially expressed at 24 hpi. Besides, we observed that LYC4 and MM differed in the expression of a large number of genes in the absence of *B. cinerea*. For instance, before *B. cinerea* inoculation, there were >8000 differentially expressed genes (approximately equal numbers of up-regulated and down-regulated genes) in the leaf samples of LYC4 compared with MM. Thus, the deciphering of the resistance mechanism is challenging but is still under investigation. Further perspectives to facilitate the study of *B. cinerea* resistance in LYC4 were proposed in **Chapter VII**. Remarkably, delayed induction of plant cell death by *B. cinerea* coupled with delayed and attenuated plant responses were revealed during LYC4 infection (**Chapter III**). Toxic compounds, possibly accumulated in the trichomes of LYC4 leaves, potentially play roles in the interaction of wild tomato-*B. cinerea* interactions as indicated by the induction of fungal detoxification genes in early infection stages on LYC4 (**Chapter III**). The other part of this thesis focused on the specific role of an important antimicrobial compound, α-tomatine, in the interaction of tomato and *B. cinerea.* The information that was available prior to this thesis project is reviewed in **Chapter IV**. So far three types of tomatinase have been reported in tomato pathogens that catalyze the hydrolysis of different glycoside bonds. As described in **Chapter VII**, tolerance mechanisms to plant antimicrobial compounds in fungal pathogens are often under transcriptional regulation. Therefore, we performed a RNA-seq analysis to reveal *B. cinerea* genes involved in tolerance to α-tomatine. We identified several α-tomatine-responsive genes including the *BcTom1* encoding GH43 tomatinase and a *GT28a* gene encoding a glycosyl transferase. We demonstrated their contribution in tolerance to α-tomatine and unveiled their role in tomato infection (**Chapter V**). This study not only filled the current knowledge gap about genes encoding tomatinase activities but also identified novel non-hydrolytic mechanisms for tolerance to α-tomatine. Besides, we believe that the RNA-seq strategy to identify transcriptional response in plant pathogens to diverse plant antimicrobial compounds can broaden our horizon of plant-microbe interactions in the future.

To study the role of α-tomatine in basal defense in tomato plants, we deleted two α-tomatine biosynthetic genes *GAME4* (Solyc12g006460) and *GAME2* (Solyc07g043410) separately by CRISRP/Cas9 genome editing (**Chapter VI**). The deletion of *GAME4* fully abolished the α-tomatine biosynthesis. However, *GAME2* knockout mutants still accumulated α-tomatine at similar concentration as the wild-type plants. This result indicates that this *GAME2* gene, which was proclaimed to encode the β_1-tomatine UDP-xylosyltransferase, is not essential for α-tomatine biosynthesis in tomato, despite the fact that it resides in a biosynthetic gene cluster with other *GAME* genes. Future studies will focus on the identification of the glycosyltransferase that is truly responsible for the last glycosylation step of α-tomatine synthesis. Besides, *GAME4*-KO plants

displayed increased susceptibility to *B. cinerea* and *Phytophthora infestans* in infection assays indicating that α-tomatine contributes to disease resistance in tomato. However, the role of α-tomatine in tomato basal defense needs to be addressed with infection of more plant pathogens as discussed in **Chapter V.**

Acknowledgements

After more than four years, I have submitted my thesis and ready for the defense. There are both negative and positive parts about my PhD thesis just like life.

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I also want to thank all the master students who have participated in my PhD project. **Ivan** and **Thanos** my first and second master student who mainly started from scratch. **Suraj** as my third master student made great contributions to my PhD project. Then **Paul**, **Aish** and **Marie-Emma** who joined in the middle and at

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Finally, I want to thank my family, my parents and my older sister. Thanks for your understanding, care and support!

Again, I sincerely wish all of you a bright future and a happy life.

About the author

Yaohua You (由曜华) was born in Liaoyang a small city in Liaoning province on 7th January 1992. His "academic career" started in the kindergarten and followed by primary school and high school which are all less than 2 km away from where he lived. After an unsuccessful College Entrance Examination, he went to Northwestern University of Agricultural and Forestry Science and Technology (NWAFU), which is more than 1700 km away from his hometown and after 4 years he obtained the bachelor degree of horticulture.

He decided to do a master thesis at the same university and conducted a research on the anthocyanin biosynthesis in red-fleshed apples in the lab of prof. dr. Pengmin Li. At the end of his master thesis, he decided to do a PhD aboard. Fortunately, with the permission of Pengmin Li he had sufficient time to pass the English language test and more importantly, he found a good supervisor dr. JAL (Jan) van Kan from Wageningen University and Research (WUR). The proposal written together with Jan van Kan was granted with a fellowship by the Chinese Scholarship council (CSC) which enabled a four year PhD contract in the Netherlands at WUR started from 2017.

From 2017 to 2022, he investigated the interaction between tomato and *Botrytis cinerea* under the supervision of Jan van Kan at the Laboratory of Phytopathology and he now has the strong motivation to continue the research regarding plant-microbe interactions after his PhD.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Yaohua You
Date: 28 June 2022
Group: Laboratory of Phytopathology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Resistance mechanisms in tomato against *Botrytis cinerea*	May 2018	1.5
► **Writing or rewriting a project proposal**		
Study on resistance mechanisms in tomato against *Botrytis cinerea*	Mar 2018	6.0
► **Writing a review or book chapter**		
You, Y. & Kan, J.A.L. van. (2020) Bitter and sweet make tomato hard to (b) eat. *New Phytologist*, 230(1), 90-100. https://doi.org/10.1111/nph.17104	2020	5.0
► **MSc courses**		
Subtotal Start-Up Phase		12.5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
EPS PhD student days 'Get2Gether', Soest (NL)	Feb 15-16 2018	0.6
EPS PhD student days 'Get2Gether', Soest (NL)	Feb 11-12 2019	0.6
► **EPS theme symposia**		
EPS Theme 2 Symposium & Willie Commelin Scholten day “Interactions between plants and biotic agents”, Amsterdam (NL)	Jan 24 2018	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten day “Interactions between plants and biotic agents”, Wageningen (NL)	Feb 1 2019	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten day “Interactions between plants and biotic agents”, Utrecht (NL)	Feb 4 2020	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten day “Interactions between plants and biotic agents”, online	Feb 9 2021	0.2
► **Lunteren Days and other national platforms**		
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 9-10 2018	0.6
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 8-9 2019	0.6
Annual Meeting 'Experimental Plant Sciences', online	Apr 12-13 2021	0.5
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 11 2022	0.3
► **Seminars (series), workshops and symposia**		
Seminar: Teemu Teeri, University of Helsinki, FI, ‘Pelargonidin in flowers - why not?' Gerbera and petunia flowers block pelargonidin biosynthesis in a different way’	Mar 15 2018	0.1
Seminar: Timothy Friesen, SDA-ARS, Fargo, ND, US, 'Genome wide association as a tool for identifying fungal effectors important in virulence'	Apr 18 2018	0.1
Seminar: Yang Wang, Nanjing Agricultural University, CN	Sep 10 2018	0.1
Seminar: Volker Lipka, Georg-August University, Göttingen, DE, ‘Live and Let Die or Live and Let Live - Interactions of Arabidopsis with fungal pathogens’	Sep 14 2018	0.1
Seminar: Tom Wood, ‘Nanopath: Utilising Nanopore sequencing for Septoria surveillance’	Sep 14 2018	0.1
Seminar: Antonio Di Pietro, University of Cordoba, ES, 'Host adaptation in the fungal cross-kingdom pathogen *Fusarium oxysporum*'	Oct 16 2018	0.1
Seminar: Jaap Wolters, Plant Breeding, WUR, NL	Feb 15 2019	0.1
Seminar: JiJie Chai, University of Cologne, DE, 'Structure, mechanism and biochemical insight of plant NLR protein'	Jun 5 2019	0.1
Seminar: David Guest, University of Sydney, AU	Sep 3 2021	0.1

	Seminar: Sophien Kamoun,The Sainsbury Laboratory, UK, 'Evolutionary dynamics of NLR immune receptors in plants'	Sep 16 2020	0.1
	Seminar: Xiao Lin, The Sainsbury Laboratory, UK, 'Dissect late blight resistance of *Solanum americanum'*	Nov 13 2020	0.1
	Seminar: Lisha Zhang, University of Tübingen, DE, 'Distinct immune sensor systems for fungal polygalacturonases in Brassicaceae'	May 21 2021	0.1
	Seminar: Ioannis Stergiopoulos, UC Davis, UK	Sep 24 2021	0.1
	Seminar: Gero Steinberg, University of Exeter, UK	Nov 5 2021	0.1
	Seminar: Frank Takken, University of Amsterdam, NL	Nov 26 2021	0.1
►	**Seminar plus**		
►	**International symposia and congresses**		
	XVIII International Congress on Molecular Plant-Microbe Interactions (IS-MPMI XVIII), Glasgow (GB)	Jul 14-18 2019	1.5
	BotrySclero2021 webinar, online	Jun 8-11 2021	0.8
►	**Presentations**		
	Poster presentation in IS-MPMI XVIII Congress 2019	Jul 16-18 2019	1.0
	Oral Presentation in BotrySclero2021 webinar	Jun 8-11 2021	1.0
	Poster presentation in 31st Fungal Genetics Conference 2022	Mar 15-20 2022	1.0
	Oral presentation in Annual Meeting 'Experimental Plant Sciences' 2022	Apr 11 2022	1.0
►	**IAB interview**		
►	**Excursions**		
	Subtotal Scientific Exposure		12.1

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
Transcription Factors and Transcriptional Regulation, Wageningen (NL)	Dec 10-12 2018	1.0
Translational Science Workshop: Taking MPMI Discoveries to the Field, online	Dec 2 2020	0.2
► **Journal club**		
Literature discussion workshop (Botrytis group, Phytopathology Laboratory)	2018-2021	1.5
► **Individual research training**		
Confocal microscopy training in Laboratory of Cell Biology	Aug 22 2018	0.2
LC-QqQ-MS training in Laboratory of Plant Physiology	Feb 22 2022	0.2
Subtotal In-Depth Studies		3.1

4) Personal Development	*date*	*cp*
► **General skill training courses**		
EPS PhD Introduction course, Wageningen (NL)	Mar 27 2018	0.3
Project and Time Management, Wageningen (NL)	Oct 30 - Dec 11 2020	1.5
Introduction to R and R Studio, online	Jan 12 - Feb 2 2021	0.9
Essentials of Scientific Writing & Presenting, online	Mar 12-26 2021	1.2
► **Organisation of meetings, PhD courses or outreach activities**		
Member of management team of the Phytopathology chair group	Oct 24- Nov 16 2021	1.0
► **Membership of EPS PhD Council**		
Subtotal Personal Development		4.9

TOTAL NUMBER OF CREDIT POINTS*	**32.6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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